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Effect of Gamma-ray upon Food Microorganisms

Studies concerning Gamma-ray Resistivity of *Escherichia coli* (III)

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Received July 19, 1958

The author has carried out studies concerning the effects of different conditions on the gamma-ray resistivity of *E. coli*. This report deals with the survival of the strain in various types of media under gamma-ray irradiation, the influence of the preservation procedure at room temperature prior to gamma-ray irradiation, and the minimum dose-rate of gamma-ray for regular survival of the strain under a weak gamma-ray field.

INTRODUCTION

In previous reports, the author has presented a simplified method for the sterilization of bacteria by gamma-ray irradiation, and has also ascertained this method to be substitutive for the usual method using bacterial solution[1,2]. However, this method should be undertaken under restricted conditions and, in case of execution of the method there still remain many problems to be clarified which are listed as follows:

(1) The difference of growth-rate of bacteria in various kinds of media irradiated with gamma-ray.

(2) Influences of various kinds of protective or chelating agents in media upon the survival of bacteria.

(3) Determination of a maximum irradiation-period for regular survival under a weak gamma-ray field. This is because bacteria in the nutrient medium could grow under gamma-ray field in this experiment.

(4) Determination of a maximum period for regular survival after solidification of the medium used until the beginning of gamma-ray irradiation.

(5) Determination of a minimum dose-rate of gamma-ray in order to obtain a semi-logarithmic relation between strain-survival and the total gamma-ray dose irradiated.

(6) Comparison of survivals of the same strain in different media by gamma-ray irradiation under identical conditions.

However, there are very few studies elucidating the above-mentioned facts. The author has therefore carried out studies on these problems, the results of which are presented herein.

EXPERIMENTAL

Escherichia coli (YC1), one of the stock cultures preserved at the School of Medicine, Keio University was used as in the previous two reports. The majority of the media used here included nutrient broth agar, (NISSAN SUISAN, PH 7.2), and occasionally two other media were used. One was a synthetic medium for *E. coli*, (minimum medium), while the other was plain agar medium, a non-nutrient as a medium containing bacteria in the gamma-ray field.

The radiation source applied was cobalt-60, 40 Curie, preserved at the School of Medicine, Keio University. All samples were irradiated at room temperature within a range of 18° to 20°C.

The time of irradiation was mostly 2 hours occasionally being adjusted in a range of 1 hour to 18.5 hours in Experiment 4.

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[1] W. Watanabe; Effect of Gamma-ray upon Food Microorganisms (I), This Bulletin, **22**, 68–77 (1958).

[2] W. Watanabe; Effect of Gamma-ray upon Food Microorganisms (II), This Bulletin, **22**, 255–261 (1958).

The bacterial concentration was always 10^2 cell/ml order. The experimental procedure is mentioned in detail in each of the following sections.

RESULTS AND DISCUSSION

(1) Experiment 1. A normal sample was treated according to the method mentioned in detail in the previous two reports. The media used in this experiment was a nutrient broth agar and the minimum medium for *E. coli* which was found by Davis, shown in Table I[3].

TABLE I. COMPONENTS OF MINIMUM MEDIUM FOR *E. coli.* (by B. D. Davis)

Component	Concentration
K_2HPO_4	0.7 %
KH_2PO_4	0.2
$Na\cdot Citrate\cdot 2\,H_2O$	0.05
$MgSO_4\cdot 7\,H_2O$	0.01
$(NH_4)_2SO_4$	0.1
Glucose	1.5
Agar	0.2~0.5

The survival-curves obtained from both broth agar and the minimum medium are shown on I-line and II-line in Figure 1. Survivals of the broth agar were higher than those of the minimum medium. Though the reasons for this result are not clear, one or two of the following reasons might be attributed;

(a) More protective or chelating agents are contained in broth agar than in the minimum medium.

(b) The growth-rate of bacteria in the broth agar is higher than in the minimum medium.

(c) Existence of recovering factors from components in the broth agar is more remarkable than that in the synthetic medium.

Secondly, the irradiated samples which contained irradiated bacteria and minimum media were divided into two groups. One group was the control, and the other group was covered with broth agar on the first minimum medium prior to incubation at 37°C. This means that the broth agar supplies nutrient components to irradiated bacteria in the synthetic medium. The results are shown on II-line and III-line in Figure 1. As it can be seen from this figure, the survivals of a double medium were higher than those of a single medium. As the result shows, bacteria irradiated with gamma-ray would survive more easily in a complete medium than in a synthetic medium, as reported by Stapleton[4]. Moreover, it would be assumed that components in the broth medium are more protective than those in a synthetic medium during gamma-ray irradiation acting as a kind of protective agent.

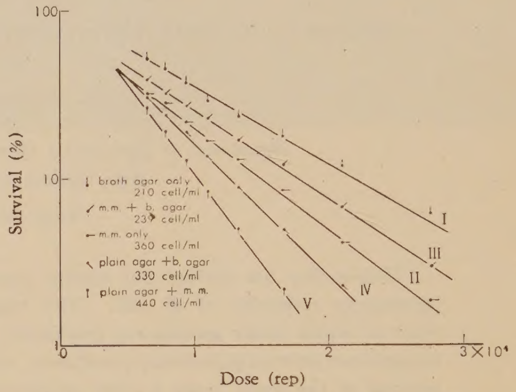

FIG. 1. Survivals of *E. coli* in Various Types of Culturing Media after Gamma-ray Irradiation.

Thirdly, bacteria were irradiated in a medium containing 1.5% agar only, non-nutrient; one group of irradiated samples was covered with the broth agar medium and another group covered with the minimum medium prior to incubation at 37°C. The results are shown on V-line and IV-line in Figure 1. Survivals obtained from both samples were less than those from other media, and survivals from a medium covered with the broth agar were higher than those covered with a minimum medium. In this experiment, bacteria in a non-nutrient medium could not grow during irradiation. Therefore, the survival-curve obtained from this media was seemed to be unadequate for comparison with that of a medium capable of growth under the same irradiation.

---

[3] B. D. Davis and E. S. Mindioly; Mutants of *E. coli* Requiring Methionin or Vitamin B-12, *J. Bacteriol.*, **60**, 17-28 (1950).

[4] G. E. Stapleton; The Role of Enzymatic Oxygen Removal in Chemical Protection against X-ray Inactivation of Bacteria, *J. Bacteriol.*, **63**, 805-811 (1952).

The growth-rate of the strains in an incubator at 37°C differed from one another. The rate in broth agar was the highest, that of a minimum medium covered with broth agar, and only a minimum medium followed in order. Bacteria irradiated in a plain agar medium was incubated for more than 48 hours after being covered with a nutrient medium, broth agar or minimum medium, until colonies developed could be calculated.

(2) Experiment 2. Normal samples were irradiated within 10 to 20 minutes after solidification of the used medium. When the period from solidification till the beginning of irradiation is more longer, bacteria in the samples is considered to begin to grow at room temperature. And, it resulted in the increase in number of the living cell of the strain, consumption of the components and accumulation of the decomposed products, thus these would be the reasons for the unaccurate survivals of the bacteria obtained.

Firstly, samples in which bacteria were contained and solidified in broth agar medium were preserved at room temperature of 18—19°C prior to irradiation for 1, 2 and 4 hours. Normal samples were irradiated immediately after solidification. The results are diagrammed in Figure 2. As it can be seen, the longer the preserved-time at room temperature, the more bacteria survived. In case of preservation for 4 hours, survivals plotted on semi-log paper were represented as a sigmoid-curve. Therefore, it would be assumed that nevertheless one cell of the strain which was locked out in a certain area of the medium was able to grow adequately at room temperasure during 4 hours, the cells were unable to be sterilized by means of gamma-ray irradiation at such a low dose-rate.

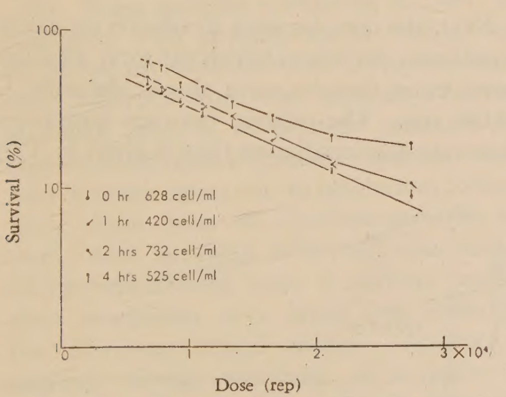

FIG. 2. Influence of Preserving Process in Room Temp. before Irradiation on Survival of *E. coli* in Nutrient Agar Medium (18–19°C).

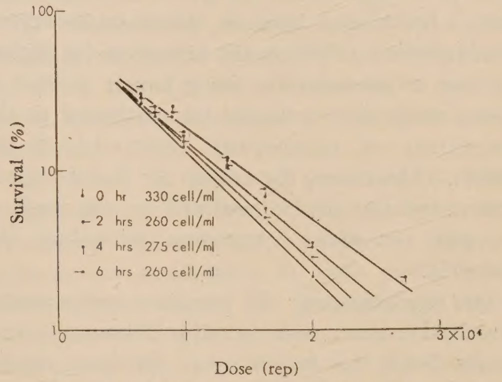

FIG. 3. Influence of Preserving Process in Room Temp. before Irradiation on Survival of *E. coli* in Non-nutrient Pure Agar Medium (18–19°C).

Next, for the sake of reference, samples which contained both bacteria and a pure agar medium, non-nutrient, were also preserved at room temperature for 2, 4 and 6 hours prior to gamma-ray irradiation. In this case, it was considered that bacteria could not be able to grow under such a condition.

After 2 hours irradiation, all samples were covered with a broth agar medium and incubated at 37°C for 48 hours. The results are diagrammed in Figure 3. As shown in the figure in this case also, the longer the sample preserved at room temperature, the more survivals exist. However, the phenomenon concerning these results should not be identical with that of the broth agar medium. Bacteria in a pure agar could not grow until covering with a broth agar medium, therefore the reason that survivals of the strain turned to be higher in case of preservation for a longer period at room temperature, should be attributed to the increment of gamma-ray resistivity of the strain. Moreover, the reason for this increased resistivity should be assumed to the lack of oxygen or some component encircling the bacteria.

(3) **Experiment 3.** In previous experiments, irradiation-time was usually 2 hours, sometimes being 1.5 hours in a previous report, and gamma-ray dose-rates were used within a range of 3,000 to 15,000 rep/h, as previously reported. In this experiment, the irradiation period was adjusted to 1, 2, 2.5, 3 and 4 hours each being irradiated at the same distance. The results are diagrammed in Figure 4. As it is seen in the figure, survivals were diagrammed on an almost straight line plotted on a semi-log paper. This shows that there is almost no time-factor in a dose-rate range of 3,000 to 15,000 rep/h, suggesting a possibility of bacteriological dosimetry by the use of this simplified method.

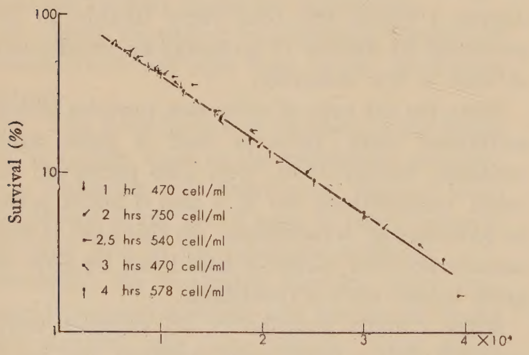

FIG. 4. Survivals of *E. coli* by Gamma-ray Irradiation in Various Irradiation Periods at the Same Distances.

(4) **Experiment 4.** A study concerning the relation between survival of the strain and gamma-ray dose-rate in a broth agar medium was attempted. Seventeen small petri-dishes were used in this experiment. The irradiation-time was 2 hours in the first run. The gamma-ray dose-rates used are shown in Table II. The results are diagrammed in Figure 5. As it can be seen, the survivals obtained from the strain irradiated at a lower dose-rate, i. e. below 4,000 rep/h, are higher than the expected value.

TABLE II. DOSE-RATES AND IRRADIATION TIMES OF SAMPLES IRRADIATED WITH COBALT-60 GAMMA-RAY AT THE DOSE OF 15,000 r.

Sample No.	Distance from Source	Dose-rate	Irradiation time	Survival
—	—cm	0 rep/h	0	100%
1	5.5	13484	1^{h}–07^{m}	29.8
2	6.3	10275	1 –28	27.4
3	7.1	8090	1 –51	29.3
4	7.9	6540	2 –18	28.8
5	8.7	5394	2 –47	31.0
6	9.5	4517	3 –19	30.3
7	10.3	3843	3 –54	31.9
8	11.2	3250	4 –37	36.4
9	12.5	2616	5 –44	44.0
10	13.7	2171	6 –55	47.1
11	15.0	1807	8 –18	57.1
12	16.2	1551	9 –40	69.5
13	17.5	1335	11 –14	77.1
14	18.7	1173	12 –47	82.4
15	20.0	1025	14 –38	79.3
16	21.2	917	16 –22	76.7
17	22.5	809	18 –33	77.9

Next, the samples were irradiated for various irradiation periods of which the total irradiated doses upon samples were almost the same, i. e. 15,000 rep. The relation between gamma-ray dose-rate and irradiation time is given in Table

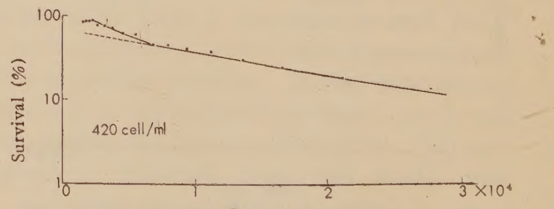

FIG. 5. Survival of *E. coli* by Gamma-ray Irradiation at the Same Time.

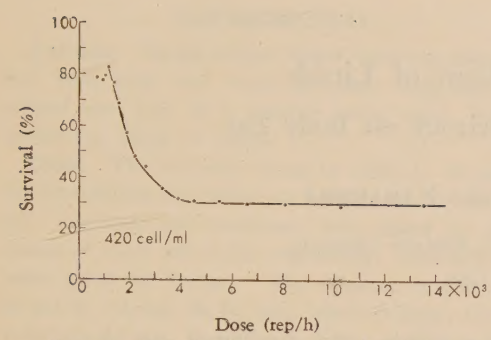

FIG. 6. Relation between Survival-ratio of *E. coli* and Dose-rate of Gamma-ray at the Dose of 15,000 r.

II. The results are also diagrammed in Figure 6. As shown in this figure, in case of gamma-ray irradiation upon *E. coli* at the dose-rate of more than 4,000 rep/h, survival-ratio had almost the same value of 30%. However, in case of gamma-ray irradiation at the dose-rate unexceeding 4,000 rep/h, the lower the dose-rate, the more bacteria survived. This means that bacteria in samples should be irradiated with gamma-ray at a dose-rate above 4,000 rep/h, and that, in case of using a dose-rate exceeding 4,000 rep/h, this simplified method can be used in bacteriological dosimetry, further indicating that there is no time-factor in a certain range of 1 hour to 4 hours.

SUMMARY

(1) When samples containing *E. coli* were irradiated with gamma-ray in various types of media, survivals obtained from each sample were different from one another. Survivals obtained from a broth agar medium were highest, those from a synthetic medium covered with a broth agar prior to incubation followed, while those from the synthetic medium were least. And, survivals of a pure agar medium which was covered with a nutrient medium after irradiation were lower than those from the above-mentioned media. Among two nutrient media, survivals of a pure agar medium covered with a broth agar were higher than those covered with a synthetic medium.

(2) Gamma-ray irradiated cells of *E. coli* markedly decreased the ability to exhibit themselves in the number of colonies in the synthetic medium as compared with broth agar medium.

(3) When *E. coli* was preserved at room temperature in a broth agar medium or a synthetic, non-nutrient, medium prior to gamma-ray irradiation, in both cases it was observed the longer the preservation period, the more cells of the strain survive. However, the reasons for each case are not identical. It is therefore necessary to apply gamma-ray irradiation upon the experimental sample immediately after solidification of the used medium.

(4) In case of using gamma-ray at dose-rates ranging between 4,000 and 14,000 rep/h, survival-ratios obtained from irradiated samples at an irradiated dose of 15,000 rep resulted to have the same value of 30%.

(5) Also, in case of applying a gamma-ray dose-rate exceeding 4,000 rep/h, there was no time-factor within a certain time-range of 1 and 4 hours.

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Studies on the Metabolism of Lipids

Part I. Influencies of Fat-deficiency on Body Fat

By Kazuo Mori and Yukihiko Nakamura

Department of Agricultural Chemistry, Hokkaido University

Received July 21, 1958

The growth of weanling rats was inhibited by administration of a fat-deficient diet and normal growth of liver was also inhibited by fat-deficiency.

The iodine value of body fat in the deficient group was considerably smaller than that of the control group, and the content of unsaturated fatty acids, especially that of linoleic acid, was found to be low.

The content of linoleate in body fat increased after the administration of linoleate but increase was not observed in case of arachidonate. Linolenate had the same affects as linoleate only when a large quantity was administered.

INTRODUCTION

In recent years much has been reported on the mechanism of lipid metabolism in mammals and its outline has become partly clear. It has been observed in vivo that the lipid is synthesized from carbohydrate. Recently Stellen and Boxer[1)] reached an important conclusion that fatty acid synthesis is a more important pathway in the utilization of glucose than the formation of glycogen. Masaro et al.[2)] fed mice with glucose which was uniformly labeled with C^{14} and clearly demonstrated the importance of lipogenesis as a pathway in glucose utilization. There exists a relation between fatty acid synthesis and diet, that is, the liver activity of converting glucose and acetate into fatty acids is also dependent upon the nutritional condition of the animal. A diet high in carbohydrate apparently provides optimum conditions for hepatic lipogenesis.

The rats which had been fed on a fat-deficient diet for a long period presented a syndrom of deficiency. Usually the rats were inspected periodically to detect symptoms of essential fatty acid deficiency. The severity of symptoms was judged by feet-scaliness, scaliness of tail and the roughness of hair coat[3,4)]. Besides these symptoms several abnormalities such as prolonged period of pregnancy[5,6,7)], still-births[8)] excessive bleeding at birth and high mortality ratio of unweaned rats were observed[9)]. It was observed that there are several differences in the constitution of body fat between normal and fat-deficient rats. The deficient syndroms disappear by administrating adequate amount of linoleate, but there is no agreement on the administrative amount among workers. The mechanism by which the essential fatty acids act in the fat-deficiency syndrom is also uncertain. The present authors intend to clarify the step in which the essential fatty acids are concerned.

[1)] D. Jr. Stellen and G. F. Boxer, *J. Biol. Chem.*, **155**, 231 (1944).

[2)] E. J. Masaro, I. L. Chaikoff and W. G. Daubem, *J. Biol. Chem.*, **179**, 1117 (1949).

[3)] Ralph T. Holman and Siret Ener, *J. Nutrition*, **53**, 411 (1954).

[4)] Ralph T. Holman and I. G. Sheldon, *Arch. Biochem. and Biophys.*, **49**, 49 (1954).

[5)] H. M. Evans, *J. Biol. Chem.*, **106**, 431 (1943).

[6)] F. C. Maeder, *Anat. Rec.*, **70**, 73 (1937).

[7)] C. G. Mackenzie, *Biochem. J.*, **33**, 935 (1939).

[8)] F. A. Kummerow, H. P. Pan and H. Hichman, *J. Nutrition*, **46**, 489 (1954).

[9)] Harry J. Deuel, Charlotte Rae Martin and Roslyn B. Aldin-Slater, *J. Nutrition*, **57**, 297 (1955).

EXPERIMENTAL

Animals Female albino Wister weanling rats which had been bred with stock diet were used and each animal was kept in a separate metal cage. Feeding period was about 11 weeks.

Diets. The diets are shown in Table I. In case of the fat-deficient diet, sugar was substituted for soy bean oil. Linoleate and linolenate were added up to an extent of 0.5% and 0.1% respectively. Diet and water were given ad libitum. The vitamine mixture contained B_1 0.1 mg., B_2 0.1 mg., niacin 0.2 mg., Ca-pantothenate 0.4 mg., B_6 0.05 mg., biotin 0.002 mg., folic acid 0.004 mg., B_{12} 0.0004 mg., inositol 2.0 mg., and choline chloride 3.0 mg. per rat per day and vitamin A 10.000 I.U. and D 1.000 I.U. per rat per week.

TABLE I. DIETS

	Sucrose	Casein	Salt	Soy Bean Oil	Linoleic Acid	Linolenic Acid
	%	%	%	%	%	%
1	74.0	20.0	6.0	—	—	—
2	74.0	20.0	6.0	—	0.5	—
3	74.0	20.0	6.0	—	0.1	—
4	74.0	20.0	6.0	—	—	0.5
5	74.0	20.0	6.0	—	—	0.1
6	69.0	20.0	6.0	5.0	—	—

Beside above components vitamin mixture was administered as noted in text.

Determination of liver carbohydrate and total lipid. Animals were killed by a blow on the head at the end of feeding period and livers were immediately taken out. They were hydrolyzed at 100° for 3 hours with 1 N-H_2SO_4, neutralyzed with NaOH, deproteined with 10% $ZnSO_4$ solution and 0.5 N-NaOH, then centrifuged. Reducing sugars were determined by the Hanes method making use of those supernatants. Total lipids were estimated by ether extraction from livers dried in vacuum over P_2O_5.

Extraction of body lipids. Total body lipids were extracted from carcasses according to the following methods. (I) Carcasses were saponified with 20% alcoholic KOH solution and lipids were separated by the usual method. (2) All carcasses were blended with petroleum ether. The lipids disolved in petroleum ether were isolated by usual methods and separated into neutral lipids and phospholipids with acetone. All solvents were removed in CO_2 stream.

Determination of fatty acids. Linoleate, linolenate and arachidonate in neutral lipids were determined spectrometrically[10], namely about 10 mg of lipids was isomerized in 21% KOH-glycol solution at 180°C for 15 minutes under nitrogen stream and their extinction coefficients were determined at wave lengths of 233, 268, 315 and 364 mμ.

RESULTS

In spite of the fact that all animals were fed for 11 weeks, no distinctive deficient symptoms appeared except roughness of hair coats, but in 2 or 3 animals the scaliness of the tail could be observed. Fig. 1 shows the body weight curves of the deficient group and the control which were fed on the diet of Table I. The increase in body weight of the deficient group appeared later than that of the control group. The average value of body weight increase in the deficient group was only 71% compared to the control group and a difference between the two groups could be found statistically.

The livers serves as a very important function in the metabolism of animals. The half-time required for body fat conversion is considered to be 16 or 17 days for saturated fatty acids and 19 or 20 days for unsaturated acids, but in the liver it is considered that only one day is needed for saturated acids and about two days for unsaturated acids. It is

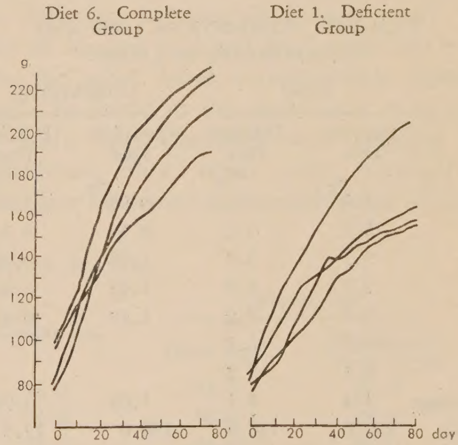

FIG. 1. Growth Curves. (I).

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[10] S. F. Herb and R. W. Riemenschneider, *Anal. Chem.*, **25**, 953 (1953).

considered that the liver is influenced in various ways by the abnormality of nutrition as the liver is very active in metabolism. For instance, under such condition enzyme activity of the liver is thought to differ from normal; this will be discussed in the following paper. Table II shows the weight of livers. The ratio of liver weight to body weight differed between two groups. The variance ratio (F value) found was 7.2 and this difference could be measured between the two groups with a probability of 0.05.

The amount of total lipids and carbohydrates in livers are shown in Table III. The total lipid content was 3.4% in the complete group. In these two groups component differences could not be found statistically.

From the above results it is shown that fat-deficiency has influence on the increase in body weight and normal growth of livers. Therefore linoleate and linolenate were administered under such conditions. Each acid was mixed in the diet in the amount of 0.5 and 0.1%. The amount of linoleate to be administered is yet not been decided among workers, i.e., Harry J. Daul[11] added 40 mg of linoleate per day, O.S. Privett[12] about 1% and P.G. Tulfpule[13] 670 mg. The present authors administered 100 mg and 20 mg per day per rat. Fig. 2 shows the influence of these fatty acids on the increase in body weight. The activity of linoleate in the increase in body weight could not be found to be the same as that of linolenate.

TABLE II. INFLUENCE ON THE WEIGHT OF LIVERS.

Complete Diet			Deficient Diet		
Liver Wt.	Body Wt.	Ratio	Liver Wt.	Body Wt.	Ratio
g.	g.	%	g.	g.	%
6.6	145	4.6	6.1	137	4.5
6.0	132	4.6	4.5	109	4.1
5.8	139	4.2	5.9	145	4.1
4.4	120	3.7	6.2	163	3.8
5.4	115	4.7	6.9	140	4.9
6.6	158	4.2	6.1	140	4.4
6.1	147	4.2	6.1	135	4.5
5.4	128	4.2	5.6	139	4.0
5.5	125	4.4	4.3	100	4.3
4.4	104	4.2	8.9	179	5.1
5.3	95	5.6	6.5	120	5.4
6.0	120	5.0	6.8	120	5.7
6.5	140	4.6	7.3	182	4.0
6.1	177	3.4	7.0	170	4.1
6.2	157	3.9	5.8	125	4.6
6.5	142	4.6	4.9	116	4.2
5.6	124	4.5	4.3	89	4.8
6.5	160	4.1	5.5	115	4.8
6.3	149	4.2	4.3	120	3.6
6.7	190	3.5	6.2	175	3.5
7.2	150	4.8	5.8	140	4.1
Average		4.34			4.55
F Value.................7.21					

TABLE III. CONTENTS OF LIPID AND CARBOHYDRATE IN LIVERS

	Lipid		Carbohydrate	
	Complete Diet	Dificient Diet	Complete Diet	Dificient Diet
	%	%	%	%
	3.0	4.1	0.58	1.00
	3.5	3.2	0.59	0.70
	3.7	3.3	1.18	0.30
	2.7	4.8	1.43	0.96
	3.5	4.2	1.49	0.84
	4.3	5.7		
	3.1	3.2		
Average	3.4	4.1	1.05	0.76
Ratio	100.0	120.6	100.0	72.3
F Value	2.64		1.66	

Next, body lipids should be taken into consideration. Table IV shows the contents of body lipids. The total content of body lipids in the fat-deficient group was identical with that of the control group, but total content of unsaturated fatty acids in the deficient group was smaller than that in the control. In the neutral lipid fraction the contents of unsaturated fatty acids were caused to become somewhat higher by the administration of linoleate,

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[11] Harry J. Daul, *J. Nutrition*, **57**, 297 (1955).

[12] O.S. Privett, *Arch. Biochem. and Biophys.*, **57**, 156 (1955).

[13] P.G. Tulfpule, *J. Biol. Chem.*, **217**, 229 (1955).

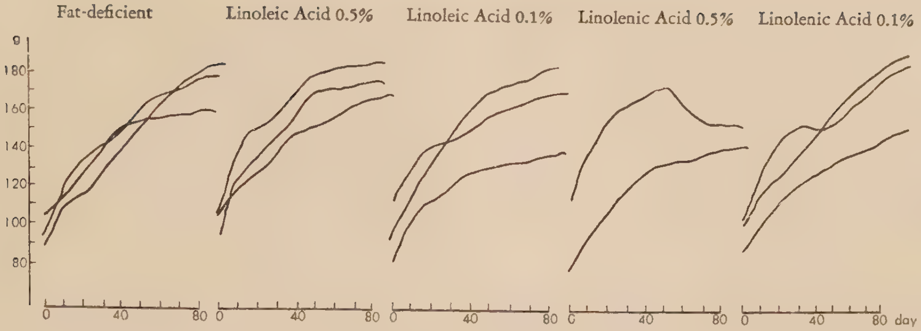

FIG. 2. Growth Curves. (II).

TABLE IV. CONTENTS OF BODY FAT

	Total Lipid	Total Fatty Acid	Total Unsaturated Fatty Acid	Neutral Fat	
				Fatty Acid*	Unsaturated* Fatty Acid
Fat-deficient	7.8%	6.0%	27.0%	76.0%	30.2%
Linoleate 0.5%	7.3			76.0	33.6
Linoleate 0.1%	7.5			70.0	33.8
Linolenate 0.5%	5.5			78.0	31.5
Linolenate 0.1%	6.8			78.0	30.9
Complete	6.8	6.4	38.0		

* These values mean the content of total fatty acids and unsaturated fatty acids in neutral fat.

but not by linolenate. However, in general, the increase in contents of unsaturated fatty acids in neutral fats was extremely small so that it was impossible to justify the conclusion that it was effected by linoleate administration.

Table V shows that the iodine values of the fat-deficient group were smaller than those of the control group. The some relationship is observed in respect to total fatty acid, total unsaturated fatty acids and neutral lipide fractions. F. A. Kummerow et al. found that the contents of oleic acid in rats which had been fed on corn oil was higher than in the fat-deficient group, this being in agreement with the present author's results. There was no difference between the groups which were fed on linoleic acid and the fat-deficient group. On the other hand, a considerable difference could be found in the fatty acid constitution of body fat. Carcasses were blended with petroleum ether, lipids were extracted and linoleate, linolenate and arachidonate of neutral

TABLE V. IODINE VALUES OF FATS

	Total Fatty Acid	Total Unsaturated Fatty Acid	Neutral Fat	Neutral Fat	
				Fatty Acid	Unsaturated Fatty Acid
Fat-deficient	60.6	85.3	54.0	65.0	82.0
Linoleate 0.5%			54.0	66.0	84.0
Linoleate 0.1%			55.0	66.0	86.0
Linolenate 0.5%			55.0	66.0	84.0
Linolenate 0.1%			57.0	67.0	84.0
Complete	81.0	98.0		81.0	

TABLE VI. CONSTITUTION OF THE BODY FATS

	Linoleate	Linolenate	Arachidonate
	%	%	%
Fat-deficient	1.39	0.57	0.21
Linoleate 0.5%	1.89	0.60	0.24
Linoleate 0.1%	1.95	0.45	0.38
Linolenate 0.5%	1.81	0.43	0.87
Linolenate 0.1%	1.37	0.64	0.34
Complete	9.24	1.48	0.44

lipid fraction were determined spectrophotometrically. The results are shown in Table VI. In the fat-deficient group the content of unsaturated fatty acids in neutral lipids was very small, especially the content of linoleate was only one-seventh that of control group. Kummerow et al. after feeding rats with corn oil recognized the content of linoleate in the fat-deficient group to be six times higher. In the present experiments the content of linoleate in the neutral lipid fraction of the group which was administered linoleate was about 30% higher than that of the fat-deficient group, being about the same with that of 0.5% and 0.1% administered groups. On the other hand when 0.5% linolenate was administered, it showed the same activity as linoleate, but did not show activity in case of 0.1% linolenate. The contents of linolenate and arachidonate in neutral lipid were hardly affected by the administration of linoleate and linolenate.

DISSCUSSION

The results of experiments to decide the period at which fat-deficient symptoms appear are not identical among various workers. Ralph T. Holman and Sheldon I. Greenberg[4] reported that in all animals being fed on fat-deficient diets the first visible symptoms appear. H. O. Kunkel and J. N. Williams[14] successfully obtained fat-deficient symptoms after 13 weeks. K. A. Kummerow et al. and O. S. Privett et al. recognized symptoms after 17 weeks and 40 weeks respectively. The present author's results concerning this point do not agree with these data, but it is convinced that the animals suffered from fat-deficiency because of their increase in body weight and liver-growth.

It is interesting to note that only the administration of linoleate exerted effects on the linoleate content in body fat whilst linolenate and arachidonate were almost not effected. James F. Mead[15] administered sodium 1-C^{14}-acetate to rats and after analysis of body fat fractions recognized that acetate was not incorporated into linoleate. They presumed that arachidonate could be synthesized by the condensation of acetate and a 18-C precursor. Gunther Steinberg and James F. Mead[16] recognized that linoleate or its derivative is incorporated with acetate into arachidonate after administration of methyl linoleate-1-C^{14} to the rats. They presumed that the arachidonate would be formed from linoleate by addition of acetate in the carboxyl end of the linoleate which was derived from linoleate, and furthermore recognized that the rate of arachidonate formation from linoleate to be relatively low. In the above-described experiments the contents of arachidonate in body fat was not effected by the administration of linoleate in spite of the increase in linoleate content. It was thus considered that the contents of arachidonate do not increase on account of the low efficiency of the linoleate to be incorporated into arachidonate.

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[14] H. O. Kunkel and J. N. Williams, *J. Biol. Chem.*, **189**, 755 (1951).

[15] James F. Mead, *J. Biol. Chem.*, **205**, 683 (1953).

[16] Gunther Steinberg and James F. Mead, *J. Biol. Chem.*, **220**, 257 (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2, p. 83~88, 1959]

On the Nitrate Reductase of Cattle Liver

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A preparation of nitrate reductase was readily obtained from cattle liver homogenate by repeated fractionation with ammonium sulfate followed by treatment with Ca phosphate gel. The activity of the reductase was exhibited by conjunction with acetaldehyde, which could be replaced by mitochondria. Some properties of the enzyme preparation are described here: opt. pH 5.8, opt. temp. 55°C, absorptions at 262 mμ and 405 mμ, sensitivity against metal and SH reagents and the participation of ferrous salt in activity. Discussions are presented on the hydrogen donating system, especially on the difference of the principle of enzymatic nitrate reduction from aldehyde and xanthine oxidases.

Vast information on nitrate reductase which plays an essential role in nitrogen assimilation have been accumulated along with the remarkably rapid progress in this field, especially concerning microorganisms.

In former years, Bernheim and Dixon[1] detected the ability of reducing nitrate in livers of many kinds of mammalia and fowl as well as in the rat muscle and demonstrated that nitrate can be reduced by merely functioning as a hydrogen acceptor of aldehyde oxidase in place of oxygen and methylene blue as in the case with milk xanthine oxidase[2]. Later on, Egami and coworkers[3] succeeded to obtain the preparation of an active enzyme from cattle liver, by mere autolysis with 2% KF at 25°C from 24 to 30 hrs. and successive precipitation at full saturation with ammonium sulfate. Using the dialysed solution of this preparation, they characterized some properties in connection with magnificient studies on *Esch. coli*[4~7]. In particular, it was stressed that the essential role of aldehyde oxidase in the nitrate reduction should be reinvestigated, because reduction was markedly inhibited by acetaldehyde, while the decoloration of methylene blue had been accelerated. On the contrary, the promotion of the nitrate reduction by acetaldehyde was delivered by Niwa[8], one of Egami's coworkers, although it was not shown clearly whether the former explanation was to be denied or not.

We[9] also recognized that the rate of nitrate reduction of the homogenate of cattle liver was enhanced to some extent by a number of organic acids and sugars, and most effectively to several times by acetaldehyde. These observations might suggest the conjunction of the nitrate reduction with relevant dehydrogenases.

EXPERIMENTAL AND RESULTS

Preparation of Enzyme

All procedures were carried out under cooling with ice unless otherwise described. The fresh liver was washed thoroughly with sterilized water, minced through a masticator and homogenized in 2 vol. of 0.05 M phosphate buffer of pH 7.2 with a Warling blender. The tissue was cut into small pieces with scissors to abridge from employing a meat grinder. After being stirred mechanically, the homogenate was poured into 3 vol. of the same buffer which had been previously warmed at about 60°C or higher, and maintained at 55°C for 5 min. Subsequently, it was rapidly cooled and then centrifuged at 3000 r.p.m. for 30 min. to remove gross materials and coagulated proteins (Fraction I). The reddish supernatant fluid was made up to 0.6 saturation by adding solid ammonium sulfate under vigorous agitation and centrifuged (Fraction II). The cake was dissolved in the buffer and the deposit at 0.2 saturation with ammonium sulfate was discarded. The enzyme was salted out from the remaining fluid by making it to a half-saturation with ammonium sulfate (Fraction III). Hereon, the precipitate was dissolved in water and the active portion between 0.25 and 0.5 saturation was gathered as above (Fraction IV). The aqueous solution of this precipitate which was pale brownish-yellow and slightly turbid, was passed through a column packed with Ca phosphate gel. After the column was washed by passing water, the enzyme was eluated with a 0.25 M phosphate buffer of pH 7.2. The white muddy eluate which first flowed down was discarded and the next eluate clear but colored was collected. The gel treatment could be performed alternatively as follows. The solution was mixed with gel suspension in water, stirred for a while and centrifuged, and this procedure repeated further two or three times using fresh gel until the supernatant became almost colorless. The absorbants combined were suspended in 0.25 M phosphate buffer, stirred rapidly and centrifuged. Finally, the enzyme was precipitated from the clear yellow eluate at 0.6 saturation with ammonium sulfate and collected. The precipitate was sucked with an aspirator through a Büchner funnel to remove moisture (Fraction V). This preparation was easily soluble in water furnishing a clear solution, the centrifugation of which at a speed higher than 15000 r.p.m. for 30 min. did not deposite any material. For the enzymatic activity, acetaldehyde was required as an indispensable hydrogen donor, contrary to the demonstration of Egami et al. concerning the inhibition caused by aldehyde. When the preparation was preserved in the cold, enzymatic activity was retained for a considerable long time,—a month or more, although the decrease in activity was inevitable. On the other hand, when the enzyme was further dried in vacuo, it rapidly changed to a glue-like substance assuming a dark brown color and became slightly soluble and almost completely deprived of activity. Even when the preparation had been frozen and dried in vacuo after the elimination of ammonium sulfate by dialysis, activity could not be maintained, though no perceptible change in appearance was observed and a small diminution of the solubility was provoked. Therefore, the preparation must be kept moist after removing as much water as possible. In this respect, the preparation of cattle liver is quite different from that of nitrate reductase of *Esch. coli*, which can be preserved in dryness with solid ammonium sulfate without loss of activity. Thus, the partial purified preparation of about 20 times' purity was obtained as a summarized in Table I.

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[1] F. Bernheim and M. Dixon, *Biochem. J.*, **22**, 125 (1928).
[2] M. Dixon and S. Thurlow, *Biochem. J.*, **18**, 989 (1924).
[3] F. Egami, S. Suzuki, M. Niwa and R. Sato, *J. Chem. Soc. Japan*, **71**, 226 (1950).
[4] F. Egami and R. Sato, *J. Chem. Soc. Japan*, **68**, 39 (1947).
[5] F. Egami and R. Sato, *J. Chem. Soc. Japan*, **69**, 160 (1948).
[6] R. Sato and F. Egami, *Bull. Chem. Soc. Japan*, **22**, 137 (1949).
[7] F. Egami and R. Sato, *J. Chem. Soc. Japan*, **70**, 397 (1949).
[8] M. Niwa, *Symposia on Enzyme Chem. (Japan)*, **7**, 68 (1952).
[9] H. Omura, *Sci. Bull. Fac. Agr. Kyushu Univ.*, **14**, 423 (1954).

Estimation of Activity

The enzymatic activity was estimated anaerobically in a Thunberg tube on account of complete inhibition by air, and expressed as the concentration of the reduction product, nitrite, in the reaction mixture. In general, the enzyme solution of 0.1 M phosphate buffer of pH 6.0 was placed in the main body of the tube with nitrate and acetaldehyde in the side arm. The total volume was made up to 10 ml for convenience of colorimetric estimation of nitrite by means of a Hitachi electric colorimeter with cells of a 10 mm path which was commonly employed. After evacuation, the contents were mixed and incubated at 40°C from 1 to 2 hours. Then, the mixture was immersed into boiling water for 3 min. to halt the reaction, cooled and deprotenized with a few drops of glacial acetic acid and

TABLE I. SUMMARY OF ENZYME PREPARATION

Fraction	Total* units	Total** protein-N, mg	Specific activity, units/ mg prot.-N	Recovery %
I. Crude extract	87609	23055	3.8	100
II. 1st ppt., 0–0.6 sat. $(NH_4)_2SO_4$,	38028	2333	16.3	43.3
III. 2nd ppt., 0.2–0.5 sat. $(NH_4)_2SO_4$,	32857	1346.6	24.4	37.5
IV. 3rd ppt., 0.25–0.5 sat. $(NH_4)_2SO_4$,	19035	1199.7	15.9	21.7
V. Final ppt. from gel eluate, 0–0.6 sat. $(NH_4)_2SO_4$,	11948	142.0	84.1	13.6

* A unit was defined as NO_2 concentration produced in the reaction mixture, μM, at 40° for 120 min

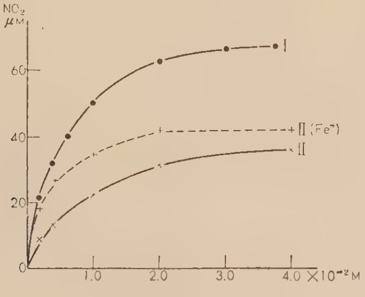
** Protein-N was determined by micro-Kjeldahl method on trichloro acetic acid-precipitate.

0.5 ml of saturated uranium acetate. The clear solution obtained after centrifugation was analyzed for nitrite with Griess's reagent.

In the experiments heretofore performed, the concentration of nitrate was 10^{-2} M. Furthermore, it was also observed that the nitrate reduction was constantly enhanced about 6 to 8 times by acetaldehyde of 10^{-3} M or higher, even with the liver homogenate containing inherent substances[9,10]. However, from Fig. 1 which shows the effects of nitrite and acetaldehyde on nitrate reduction, it will be evident that in both cases the quantities of nitrite were approximately constant at concentrations above 2×10^{-2} M, respectively. So, these amounts were applied thereafter.

The dependence of activity on the dose of the enzyme preparation and direct proportionality of reduction to reaction time were also recognized (Fig. 2). Five to 15 μM of nitrite solution was most suitable for our colorimetric estimation and the activity of the enzyme preparation was varied with the original liver, as described below. It was therefore desirable to check the amounts of enzyme preparation and the reaction time previously so as to produce moderate quantities of nitrite. Alternatively, in case of disregarding such precautions, the reaction mixture should be diluted prior to coloration. At any rate, the reaction-time should be restricted within 2 hrs.

From preliminary experiments on the purification of the enzyme, it had been noticed that maintaining the initial homogenate at 55°C for 5 min. did not result in weakening activity. As shown in Fig. 3, it was realized that the nitrate reduction catalysed by cell free preparation also proceeded most actively around 55°C. However, for the sake of practical convenience, estimation of the activity was carried out at 40°C because the reaction mixture was vigorously boiled up at a higher temperature in vacuo.



Conc. of NO_3 or AA

FIG. 1. Effect of Concentration of NO_3 and Acetaldehyde on the Nitrate Reduction.

I: NO_3 concentration

II: Acetaldehyde concentration

Enzyme preparation, 20 mg; Reaction time, 60 min. The enzyme preparation used in I was different from that used in II.

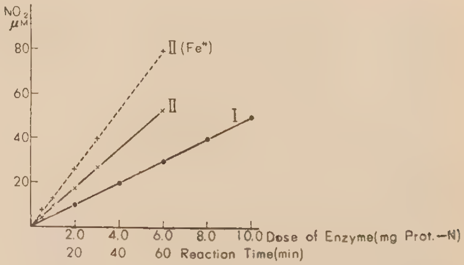


Fig. 2. Effect of Dose of Enzyme Preparation and Reaction Time on the Nitrate Reduction.

I: Dose of Enzyme Preparation (Reaction time, 60 min.)

II: Reaction Time. (Enzyme preparation, 25 mg)

The enzyme preparation used in I was different from that used in II.

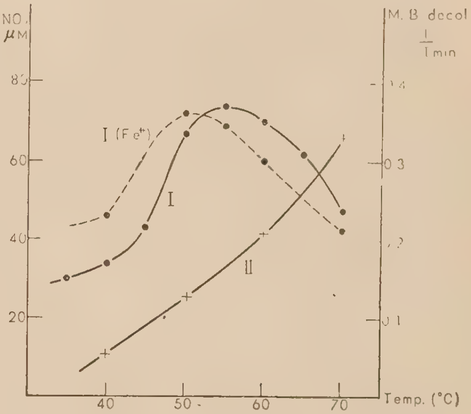


FIG. 3. Effect of Temperature on the Nitrate Reductase Activity and the Aldehyde Oxidase Activity of the Enzyme Preparation.

I: Nitrate Reductase Activity

II: Aldehyde Oxidase Activity

Enzyme preparation 20 mg; Reaction time, 60 min.

pH Optimum

The effect of pH on activity was determined by phosphate and acetate buffers of various values. As illustrated in Fig. 4, the optimum pH is presented at about 5.8, which agrees, on the whole, with that of the crude homogenate determined without external addition of acetaldehyde[9]. However, this value is dis-

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[10] H. Omura, *J. Fac. Agr. Kyushu Univ.*, **10**, 365 (1956).

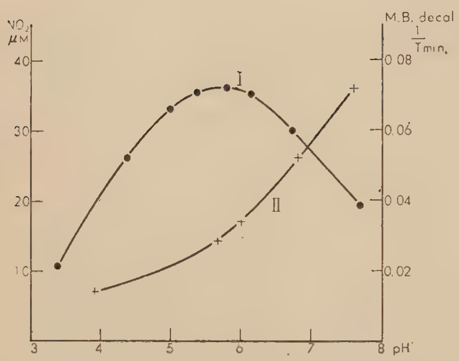

FIG. 4. Effect of pH on the Nitrate Reductase Activity and the Xanthine Oxidase Activity of the Enzyme Preparation.

I: Nitrate Reductase Activity
II: Xanthine Oxidase Activity
Enzyme preparation, 20 mg; Reaction time, 60 min.

crepant to the value reported by Egami et al. about 7.6, on their cattle liver preparation[3]. It also differs from that of TPN-nitrate reductase of *Neurospora*, 7.0, evidenced by Nason and Evans[11], as well as that of *Esch. coli*'s enzyme coupled with formic dehydrogenase, 7.4, studied by the school of Egami[4].

Effect of Inhibitors

The influence of some inhibitors is summarized in Table II. In these works, the enzyme solution was mixed simultaneously with nitrate and an inhibitor to be tested after evacuation. The enzyme was sensitive to metal combining reagents such as KCN, thiourea, EDTA and 8-hydroxyquinolin. Among these, KCN was most effective. On the other hand, reduction was completely obstructed by $CuSO_4$ at a concentration as low as 10^{-5} M, whereas a half-retardation resulted by monoiod-acetic acid of 10^{-2} M. These results might possibly suggest the participation of metal and SH groups in the enzymatic reduction of nitrate. In addition, some inhibitions about 50% were also caused by NaF at the concentration of 10^{-2} M.

TABLE II. EFFECT OF INHIBITORS

Inhibitor	Conc. M	NO_2 formed, μM	Inhibition %	Time of M.B. decol., min.
None		51.6		4.5
KCN	10^{-2}	7.5	85.5	120
	10^{-3}	37.2	28.0	—
Thiourea	10^{-2}	33.6	34.9	5
EDTA	10^{-2}	38.7	25.0	—
8-Hydroxyquinoline	5×10^{-3}	41.3	20.0	—
Monoiod-acetic acid	10^{-2}	25.8	50.0	7
	5×10^{-3}	36.1	30.0	—
$CuSO_4$	10^{-5}	0	100.0	∞
NaF	10^{-2}	23.7	54.0	8

Enzyme preparation, 20 mg; Reaction time, 120 min.; ∞ indicates that no decoloration occurred in 120 min.

Absorption Spectrum

The absorption spectrum was determined with a Beckman Model DU spectrophotometer. In the visible range, the enzyme preparation was dissolved in M/15 phosphate buffer of pH 6.0 to form a solution of 10 mg per ml and submitted to estimation. On the other hand, against ultraviolet light, the solution diluted 10-times further was served for reading the optical densities. As illustrated in Fig. 5, the spectrum has two peaks, the main one at 262 mμ and the second at 405 mμ, and two bottoms of the concaves at 246 mμ and 371 mμ.

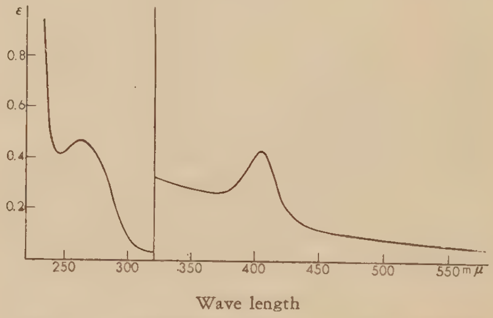

FIG. 5. Absorption Spectrum of the Enzyme Preparation.

Effect of Metals

In preparation of the enzyme, a sudden depression of activity was often experienced after salting out with ammonium sulfate. An attempt to restore activity was accomplished by the addition of ferrous salts, although no special means had been applied beforehand in order to remove metals from the enzyme preparation and water further by using ordinary laboratory distilled water (Figs. 2 and 3). In addition, it was observed that the activity was conversely inactivated by the same ion at a higher temperature giving rise to the fall of the opt. temperature to 50°C as shown in Fig. 3. However, some other metals including ferric ion were proved unsuccessful to recover activity, while zinc had an ability to inhibit the reduction, and the effect of

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[11] A. Nason and H. J. Evans, *J. Biol. Chem.*, **202**, 655 (1953).

TABLE III. EFFECT OF METALS

Metal added, 10^{-3}M	None	Fe^{++}	Fe^{+++}	$MoO_4^{=}$	Zn^{++}	Mn^{++}	Mg^{++}	Co^{++}
NO_2 formed, μM	108	139	112	105	75	91	101	102

Enzyme preparation, 20 mg, ; Reaction time, 120 min.

manganous salt was vague. The most remarkable hindrance caused by copper is already cited in Table II.

From Fig. 2, it is inferable that the reactivating function of ferrous ion may be instantaneous, since the enzymatic nitrite formation from nitrate was enhanced in a period of 5 min. after the beginning of reduction. In these works, both nitrate and metal were added concurrently into the enzyme solution to start the reaction.

The enzymatic activity in acetate buffer was higher than that in phosphate under the same conditions. On the contrary, as shown in Table IV, the degree of activation by ferrous salt was more effective in case of the later than that in the former.

TABLE IV. EFFECT OF BUFFERS

Buffer, pH 6.0	Acetate		Phosphate	
Fe^{++}, 10^{-3} M	None	Added	None	Added
NO_2 formed, μM	9.6	13.8	5.0	10.8

Enzyme preparation, 10 mg ; Reaction time, 60 min.

On the other hand, as mentioned above, the inhibition by metal chelating reagents such as KCN and EDTA suggested the essential role of metal in the enzymatic nitrate reduction even without the supplementation of ferrous salt. Therefore, it is assumed that there might at least be two kinds of metals which participate in the activity of nitrate reductase of liver tissue, the one combined tightly to the enzyme protein and the other loosely, and that the later is ferrous ion removable during the precipitation with ammonium sulfate, whereas the former had not yet been characterized.

Hydrogen Donating System

As referred above, regarding the nitrate reduction of the homogenate, any hydrogen donor was not required, for the liver contains the inherent substrates. However, activity was accelerated by several organic acids and sugars as well as acetaldehyde. Therefore, it was presumed that the relevant dehydrogenases take part in the reaction. On the other hand, the purified preparation could not catalize the nitrate reduction unless acetaldehyde had been added, while other above-substances had no effect. When nitrate was substituted with methylene blue, decoloration was brought about being due to the function of aldehyde oxidase. Furthermore, the observation that the enzymatic bleaching of dye is also delayed by inhibitors of nitrate reductase is cited in Table II in which the activity was estimated under the same conditions with the nitrate reduction except that the dye of 10^{-4} M final concentration was employed. Although the intimate connection between both activities was recognized, any linear relationship was not noticed in various preparations which were prepared one by one through the definite procedure, as shown in Table V. The results indicated the reason why the enzyme solution should be prepared in a lump for each test. As illustrated in Fig. 3, it is observed that the velocity of decoloration is faster at 70°C than at 55°C, the optimum temperature of nitrate reductase.

Concerning xanthine oxidase, a clue impling that nitrate reductase may be different from xanthine oxidase itself was obtained on the basis of the study regarding inhibition with some purine derivatives[12]. Figures in Table VI may also support this conjecture. The power to reduce both nitrate and methylene blue was estimated with 2×10^{-3} M acetaldehyde, xanthine and hypoxanthine as the substrate. Though a function of purines acting as the hydrogen donor could be evidenced, the amounts of nitrite formed were much less than those with aldehyde, whereas the rates of decoloration were entirely contrary. Therefore, it appeared probable that

TABLE V. ENZYME ACTIVITY OF SOME PREPARATIONS

Prep. No.	1	2	3	4	5	6	7	8	9	10
NO_2 formed, μM	66.8	56.0	43.0	18.0	16.0	16.0	15.4	13.1	8.3	4.1
Time of M. B. decol. min.	2	8.5	18	80	3	20	5.5	—	30	6

Enzyme preparation, 10 mg ; Reaction time, 120 min.

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12) H. Omura, This Bulletin, **22**, 387 (1958).

TABLE VI. EFFECT OF XANTHINE AND HYPOXANTHINE

Substrate	Acetaldehyde	Xanthine	Hypoxanthine	Acetaldehyde and xanthine	Acetaldehyde and hypoxanthine
NO_2 formed, μM	26.8	3.4	4.5	4.1	6.3
Time of M.B. decol. min.	30	18	16	20	25

Enzyme preparation, 10 mg; Reaction time, 120 min.

nitrate reductase might be inhibited by the substrate, xanthine and hypoxanthine, while the delivery of hydrogen from purines had been vigorously performed, which coincided with the observation on mouse liver homogenate and was discussed by Hofstee[13] on xanthine oxidase. In addition, an examination of Fig. 4 revealed that the activity of xanthine oxidase increases in the alkaline side above the opt. pH of nitrate reductase.

Thus, these results presumably give support to the assumption that the nitrate reduction might be catalized by a special enzyme or an active centre different from aldehyde and xanthine oxidases, notwithstanding that oxidase is the essential hydrogen donating system.

Nitrate reductase of *Esch. coli* was found to conjugate with formic dehydrogenase and the efficiency of FAD and methylene blue as the hydrogen carrier was also observed. Furthermore, it was established that FADH and leuco methylene blue can behave as an immediate substrate of coli enzyme and TPNH as that in case of *Neurospora*. As for the liver enzyme, however, neither the availability of formic acid nor the activation of the dialysed enzyme solution by FAD and methylene blue was demonstrated. It is known that FAD is included as the coenzyme of aldehyde and xanthine oxidases and that special means must be taken to remove FAD from the enzymes. Hence, there is another problem to be discussed whether the flavine compounds are concerned as the prosthetic group of nitrate reductase of liver or not.

Then, with the object to elucidate the intracellular behaviour of the enzyme, the mitochondria were isolated by the differential centrifuge method of Hogeboom, Schneider and Pallade[14] and suspended in the isotonic sucrose solution so as to contain particles from 0.3 g fresh liver per ml. The effect of 1 ml mitochondrial suspension on nitrate reductase is indicated in Table VII. Ability of the mitochondria to reduce nitrate was scarcely testified and could not be enhanced by aldehyde. However, mitochondria induced nitrite formation with reductase preparation without acetaldehyde, which lacks such ability by itself. In other words, nitrate reductase can accept hydrogen from the mitochondria in place of aldehyde within the liver cell. The function of the mitochondria might be ascribed to endogenous activity of the particles and explain the acceleration of nitrate reduction of the liver homogenate by some organic acids such as glutamic and succinic acids which can not function as a hydrogen donor of the enzyme preparation employed in this study, since the mitochondria include several of these dehydrogenases. The details will be reported later[15].

TABLE VII. EFFECT OF MITOCHONDRIA

Nitrate reductase	−	−	+	+	+	+
Mitochondria	+	+	−	+	+	−
Acetaldehyde	−	+	+	−	+	−
NO_2 formed, μM	0.8	0.9	3.9	4.5	7.2	0

Enzyme preparation, 10 mg; Reaction time, 60 min.

Acknowledgement This work was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education.

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[14] C. H. Hogeboom, W. C. Schneider and G. E. Pallade, *J. Biol. Chem.*, **172**, 619 (1948).

[15] H. Omura, Enzymologia, in press.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2, p. 89~94, 1959]

On the Migration Distance in Paper-electrophoresis
Part VII. Studies on the Migration Distance of Dyes (2)

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The migration distance of several scores of dyes in 30% and 4% acetic acid solutions in a horizontal-type apparatus permitting evaporation was measured. In these occasions, an extremely relationship was found to exist between the degree of influence of osmotic flow into filterpaper due to evaporation from the paper surface and the displacement ratios obtained from adsorption analysis with the same filterpaper.

The authors have already reported that amino acids[1,2] and serum proteins[3] show a decrease in migration velocities approaching a logarithmic decrement in the horizontal-type apparatus permitting evaporation and simultaneously found that the relative distance ratios of these substances, named MG values[4~7], are constant values regardless of voltage, current, time of electrophoresis, dropped positions and length of filterpaper. Further in the case of amino acids[1,4] and serum proteins[3], it was found that parallel straight-line graphs could be plotted between migration distance and dropped positions, and the MG value was proved to assume a constant value regardless of the above-conditions by means of analysing the above-graphs[5,6]. However, this fact was not the case with all of the other substances for throughout the experiments on several scores of dyes in citrate buffer[8], the graphs of which were not all found be parallel to each other.

Experimental results of migration distance of these dyes placed in 30% and 4% acetic acid solutions are presented in this paper.

EXPERIMENT AND DISCUSSION

I. Materials and method

(1) Aqueous solutions 0.1, 0.5 and 1% of the following 31 different dyes were used.

(i) Triphenylmethane dyes: kiton blue A, pontacyl green BL, acid blue VS, wool violet 4BN, brom phenol blue, rohdamine B, malachite green, uranine, fuchsine, methyl violet, crystal violet, eosine B, rose bengal B.

(ii) Diphenyl methane dyes: auramine.

(iii) Dis azo dyes: congo red, bismarck brown R.

(iv) Azo dyes: new coccine, tartrazine, orange G, sunset yellow, methyl orange, amaranth, chrysoidine ES, orange II, ponseau R, yellow AB.

(v) Nitro dyes: naphthol yellow S.

(vi) Anthraquinon dyes: alizarin red S.

(vii) Indigo dyes: indigo carmine.

(viii) Azine dyes: neutral red.

(ix) Thiazine dyes: methylene blue.

In addition, A 2% aqueous solution of dextran and 1.0, 0.5 and 0.1% solutions of glycine, histidine, arginine, lysine, glutamic acid and aspartic acid were used.

(2) The apparatus was of the horizontal-type permitting evaporation as shown in Fig. 1. For part D

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[1] K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 181 (1958).

[2] K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 186 (1958).

[3] K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 275 (1958).

[4] K. Sakamoto and K. Tateoka, *J. Agr. Chem. Soc. Japan*, **30**, 463 (1956).

[5] K. Sakamoto and K. Tateoka, This Bulletin, **20**, 98 (1956).

[6] K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 279 (1958).

[7] K. Sakamoto and K. Saito, This Bulletin, **22**, 55 (1958).

[8] K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 390 (1958).

in the figure Tôyô Filterpaper No. 50, 2×40 cm and 2×35 cm, and for part C 30% and 4% acetic acid solution were used respectively. Twenty minutes after the beginning of current application, the test solution was dropped on the filterpaper and subsequently subjected to electrophoresis under a constant voltage, and the current measured at an interval of 10 minutes, the arithmetical mean being assumed as the average current. Then after judging the center of deep colored specks as the positions of the dyes, migration distance was measured.

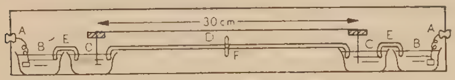


FIG. 1. Apparatus.

A) electrode B) KCl solution C) acetic acid solution
D) filterpaper E) agar bridge F) glass tube

II. Results and discussion

(1) Adsorption analysis: As reported previously[1,3,8], when the substance migrates in the horizontal-type apparatus permitting evaporation, evaporation due to Joule's heat increases and migration velocity is conspicuously influenced by the osmotic flow into filterpaper. As a means of observing the influence of this osmotic flow, the adsorption analysis was conducted with the filterpaper in the apparatus shown in Fig. 2.

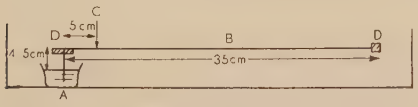


FIG. 2. Apparatus of Adsorption Analysis.

A) acetic acid solution B) filterpaper
C) dropped point of dye solution D) holding rod

R values, displacement ratios, calculated out according to the following definition are shown in Table I.

$$R=\frac{\text{displacement of substance from its dropped position}}{\text{distance of the soaked up front of acetic acid solution from dropped position of substance}}$$

The difference in concentration of dye solutions and their volume to be dropped were examined in order to obtain a variation of R values. Concretely, to obtain R values in the former test, a portion of 1/400 cc of each dye solution of 1%, 0.5% and 0.1% respectively, was examined and in the latter test, portions of 1/100 cc, 1/400 cc and 1/800 cc of 1% dye solution were examined respectively. In this case the R values of dextran, glycine, histidine, arginine, lysine, glutamic acid and aspartic acid were also obtained. As it is obvious from the table, when 30% acetic acid solution was used R values for various dyes were roughly above 0.7, regardless of the differences of concentration and dropped volume, whereas when 4% acetic acid solution was used, R values in general, showed a decrease along with the decrement of concentration and dropped volume. However in both cases R values of amino acids resulted to be 1.00, as displacement proceeded with the soaked up front. Hence no particular relation could be observed between R values and the chemical structures. Varieties of R values of dyes thus obtained are considered to have been caused by the difference in adsorption to filterpaper of these dyes.

R values obtained from adsorption analysis are the values resulting from soaking up the acetic acid solution into dried filterpaper which is not to be considered as an osmotic flow into filterpaper in paper-electrophoresis; however, a very close relation is to be seen between both of them which has considerable influence upon MG values. This relation will be described in detail later on.

Here in Table I, in order to obtain MG values dyes are classified into groups for convenience viz, in case of 30% acetic acid solution, group A, B, C, D, E and F, while in case of 4% acetic acid solution, group A′, B′, C′, D′, E′, F′, G′, H′ and I′ according to their R values.

(2) Migration distance of dyes: In acetic acid solutions of 30% and 4% respectively, each of the groups mentioned above was dropped on the center and at a point, 8 cm distant from both the anode and cathode side of the filterpaper, shown in Fig. 1 and the migration distance measured in 40, 80 and 120 minutes, respectively. As reported previously it is seen that straight line graphs[1,3,8] plotted between the axes representing migration distance and dropped positions were entirely not parallel, and, it is especially significant that dyes having lines parallel with dextran are limited in their number, where the dyes having the same R value, as dextran, 1.00, in adsorption analysis belong to. In order to avoid complexity, test results will be given in Fig. 3 selecting one kind of dye from each group already classified by adsorption analysis with acetic acid solutions. On the other hand, the authors, having recognized that variation of concentration and dropped volume of dye solutions causes change of R values in the adsorption analysis, applied electrophoresis dropping

each dye on the above-mentioned point of the filterpaper, varying concentration and dropped volume. The results obtained from some dyes are shown in Fig. 4.

As it is obvious from the figures, varieties of R values in adsorption analysis are represented in terms of varieties of angles each formed by straight line graphs with abscissa. As each dye is under influence of osmotic flow into filterpaper, to a different extent it is misleading

TABLE I. THE INFLUENCE OF CONCENTRATIONS AND DROPPED VOLUME ON THE R VALUES OF DYES AND AMINO ACIDS IN ADSORPTION ANALYSIS

(I) in 30% acetic acid solution
(II) in 4% acetic acid solution
Tôyô filterpaper No. 50, 25~29°C, 66~85 R. H.

(I)

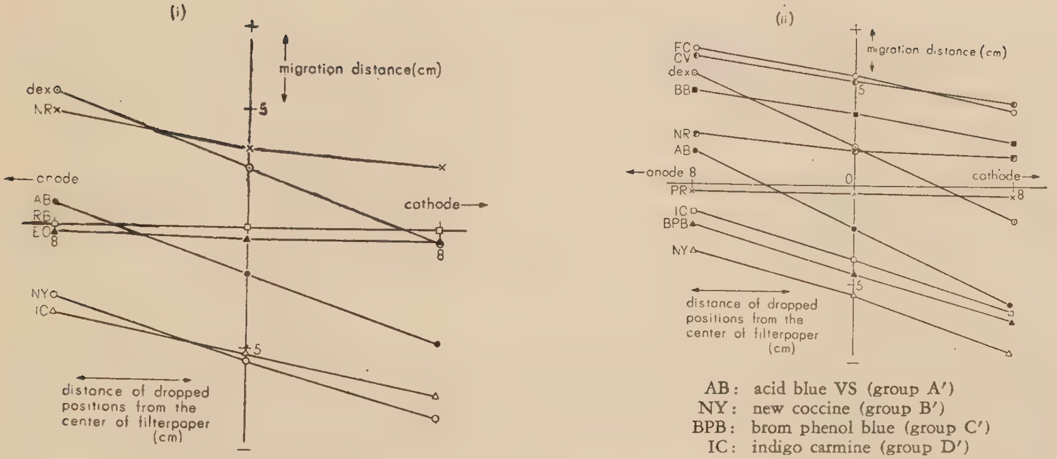
concentration (%)	0.1	0.5	1.0	1.0	1.0	group
dropped volume (cc)	1/400	1/400	1/800	1/100	1/400	
dye and amino acid						
acid blue VS	1.00	1.00	1.00	1.00	1.00	A
orange G	1.00	1.00	1.00	1.00	1.00	A
rohdamine B	0.96	1.00	1.00	1.00	1.00	A
pontacyl green BL	0.96	1.00	1.00	1.00	1.00	A
new coccine	0.96	1.00	1.00	1.00	1.00	A
malachite green	0.92	1.00	1.00	1.00	1.00	A
sunset yellow	0.91	1.00	1.00	1.00	1.00	A
wool violet 4 BN	0.85	1.00	1.00	1.00	1.00	A
kiton blue A	0.80	0.91	0.93	1.00	1.00	A
brom phenol blue	0.93	0.94	0.95	1.00	0.98	B
naphthol yellow S	0.89	0.93	0.95	0.97	0.97	B
amaranth	0.88	0.90	0.94	0.95	0.95	B
crystal violet	0.86	0.95	0.90	0.95	0.95	B
tartrazine	0.91	0.94	0.94	0.88	0.94	B
methyl orange	0.92	0.92	0.92	0.92	0.92	B
fuchsine	0.88	0.92	0.89	0.95	0.92	B
bismarck brown R	0.58	0.89	0.87	0.94	0.91	B
alizarine red S	0.87	0.90	0.90	0.89	0.90	B
indigo carmine	0.85	0.85	0.82	0.97	0.89	C
methyl violet	0.67	0.79	0.80	0.98	0.86	C
auramine	0.83	0.84	0.83	0.87	0.85	C
methylen blue	0.67	0.79	0.80	0.98	0.85	C
orange II	0.98	0.90	0.65	0.77	0.82	C
uranine	0.79	0.80	0.79	0.80	0.81	C
chrysoidine ES	0.70	0.71	0.71	0.88	0.77	D
neutral red	0.48	0.55	0.51	0.69	0.59	D
ponseau R	0.33	0.35	0.35	0.45	0.40	E
eosine B	0.18	0.20	0.22	0.23	0.23	E
congo red	0	0	0	0	0	F
rose bengale B	0	0	0	0	0	F
yellow AB	0	0	0	0	0	F
glycine	1.00	1.00	1.00	1.00	1.00	
histidine	1.00	1.00	1.00	1.00	1.00	
arginine	1.00	1.00	1.00	1.00	1.00	
lysine	1.00	1.00	1.00	1.00	1.00	
glutamic acid	1.00	1.00	1.00	1.00	1.00	
aspartic acid	1.00	1.00	1.00	1.00	1.00	

(II)

concentration (%)	1.0	0.5	1.0	1.0	1.0	group
dropped volume (cc)	1/400	1/400	1/800	1/100	1/400	
dye and amino acid						
acid blue VS	0.35	0.98	0.97	1.00	0.98	A′
wool violet 4 BN	0.41	0.70	0.79	0.99	0.96	A′
orange G	0.80	0.90	0.90	0.90	0.90	B′
new coccine	0.79	0.89	0.87	0.93	0.88	B′
pontacyl green BL	0.61	0.73	0.81	0.91	0.86	B′
naphthol yellow S	0.67	0.73	0.71	0.85	0.83	B′
sunset yellow	0.54	0.59	0.72	0.96	0.79	B′
tartrazine	0.45	0.68	0.68	0.86	0.75	C′
kiton blue A	0.36	0.65	0.63	0.81	0.74	C′
amaranth	0.41	0.66	0.67	0.81	0.74	C′
brom phenol blue	0.61	0.69	0.68	0.76	0.72	C′
indigo carmine	0.32	0.57	0.57	0.74	0.67	D′
alizarine red S	0.46	0.57	0.58	0.75	0.66	D′
malachite green	0.53	0.58	0.52	0.72	0.64	D′
methyl orange	0.62	0.60	0.59	0.60	0.61	D′
rohdamine B	0.39	0.58	0.53	0.69	0.58	E′
auramine	0.40	0.53	0.50	0.60	0.57	E′
fuchsine	0.36	0.46	0.43	0.62	0.53	E′
methylen blue	0.14	0.28	0.30	0.59	0.41	F′
orange II	0.72	0.75	0.39	0.36	0.41	F′
bismarck brown R	0.23	0.40	0.37	0.48	0.40	F′
chrysoidine ES	0.19	0.36	0.31	0.55	0.37	F′
methyl violet	0.10	0.35	0.28	0.46	0.32	G′
crystal violet	0.08	0.35	0.26	0.34	0.29	G′
uranine	0.33	0.27	0.28	0.21	0.26	H′
neutral red	0.05	0.12	0.10	0.20	0.16	H′
ponseau R	0.03	0.04	0.03	0.07	0.04	I′
eosine B	0	0	0	0	0	I′
congo red	0	0	0	0	0	I′
rose bengale B	0	0	0	0	0	I′
yellow AB	0	0	0	0	0	I′
glycine	1.00	1.00	1.00	1.00	1.00	
histidine	1.00	1.00	1.00	1.00	1.00	
arginine	1.00	1.00	1.00	1.00	1.00	
lysine	1.00	1.00	1.00	1.00	1.00	
glutamic acid	1.00	1.00	1.00	1.00	1.00	
aspartic acid	1.00	1.00	1.00	1.00	1.00	

to realize facts judging migration velocity and the direction of each dye and its electrification from the position separated after migration, when any given dye is dropped on a given point of filterpaper—even on the central point in the horizontal-type apparatus evaporation. Such being the case, in an apparatus of this type the dyes which enable correction of displacement of the original point with dextran should be limited to those whose straight line graphs between the axes of migration distance and dropped positions are parallel with graphs provided by dextran. However in consideration of the influence of electro-osmosis, even in case the substance dropped on the central point of filterpaper is displaced a little toward the anode, it can be considered to be actually migrated.

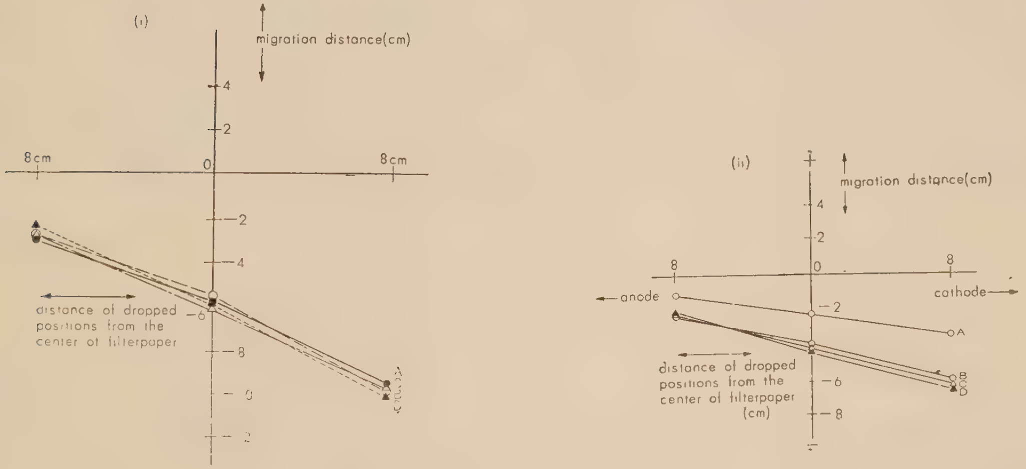
The results obtained after allowing each of the above dyes to migrate in acetic acid of 30% and 4% each are classified by their series and radicals as follows:



(i) in 30% acetic acid solution
AB: acid blue VS (group A)
NY: naphthol yellow S (group B)
IC: indigo carmine (group C)
NR: neutral red (group D)
EO: eosine B (group E)
RB;: rose bengale B (group F)
dex: dextran
0.21 mA/cm, 6.5 v/cm, 3 hours

AB: acid blue VS (group A′)
NY: new coccine (group B′)
BPB: brom phenol blue (group C′)
IC: indigo carmine (group D′)
FC: fuchsine (group E′)
BB: bismarck brown R (group F′)
CV: crystal violet (group G′)
NR: neutral red (group H′)
PR: ponseau R (group I′)
dex: dextran
0.13 mA/cm, 6.5 v/cm, 3 hours
dropped quantity: 25 γ
length of filterpaper: 40 cm in the horizontal-type apparatus permitting evaporation

FIG. 3. The Relationship between Migration Distance and Dropped Positions



(i) orange G

(ii) wool violet 4 BN the concentration and dropped volume of dye solutions are as follows

A: 2.5γ (0.1%, 1/400 cc) B: 12.5 γ (0.5%, 1/400 cc)
C: 25γ (1%, 1/400 cc) D: 100 γ (1%, 1/100 cc)

6.5 v/cm, 0.22 mA/cm, 3 hours, in 30% acetic acid solution, length of filterpaper: 40 cm, in the horizontal-type apparatus permitting evaporation.

FIG. 4. The Influence of Dropped Quantity on the Graphs showing the Relation between Migration Distance and Dropped Positions

(i) Acid blue VS, pontacyl green BL, wool violet 4BN which are sulfonic acid salts belonging to triphenylmethane dyes: All migrate toward the anode. Brom phenol blue, kiton blue A, sulfonic acids with hydroxyl group belonging to triphenylmethane dyes: These also migrate toward the anode.

(ii) Eosine which is phenolate with carboxyl group belonging to triphenylmethane dyes; uranine, with hydroxyl groups belonging to triphenylmethane dyes: Either of them migrate though slightly toward the anode.

(iii) New coccine, orange G, tartrazine, sunset yellow, amaranth, orange II, ponseau R, which are sulfonic acid salts with hydroxyl groups belonging to azo dyes: Each migrate toward the anode. Methyl orange, tertiary amine and sulfonic acid salt belonging to azo dyes: These migrate toward the anode.

(iv) Indigo carmine, sulfonic acid salt belonging to indigo dyes: These migrate toward the anode.

(v) Naphthol yellow S, sulfonic acid belonging to nitro dyes: These migrate toward the anode.

(vi) Alizarine red S, sulfonic acid salt with hydroxyl groups belonging to anthraquinon dyes: These dyes migrate toward the anode.

(vii) The following amine dyes do not belong to group A or group A′ in the adsorption analysis and are displaced toward the cathode when they are dropped on the central point, consequently making it impossible to find the accurate migration direction, but are further separated toward the cathode than dyes of (i)–(vi).

a) Triphenylmethane dyes: malachite green, rohdamine B, fuchsine, methyl violet, crystal violet.
b) Diphenyl methane dyes: auramine.
c) Thiazine dyes: methylen blue.
d) Azine dyes: neutral red.
e) Azo dyes: chrysoidine ES, bismarck brown R.

(viii) All of the following dyes are not displaced from the original point on filterpaper.

a) Dis azo dyes: congo red.
b) Azo dyes: yellow AB.
c) Triphenylmethane dyes: rose bengale B.

Thus systematic migration resulted to be in accordance with the chemical structure and the dissociation state of these dyes in the above solutions is acknowledged.

SUMMARY

Migration distance of 31 kinds of dyes in the horizontal type apparatus permitting evaporation with acetic acid solutions of 30% and 4% were measured. At the same time, adsorption analysis with the above acetic acid solutions, disclosed the existance of a close relation between the extent of influence of osmotic flow into filterpaper due to the evaporation from the paper surface and the displacement ratio (R values) in adsorption analysis with the same filterpaper. In consideration with these facts migration directions of dyes were examined in the above solution.

Acknowledgement In concluding this paper, the authors would like to express their cordial appreciation to Prof. Y. Ôshima of Kyushu University for his constant guidance. Thanks are also due to San-ei Chemical Manufacturing Co. Ltd. for kindly supplying the dyes for this work.

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On the Migration Distance in Paper-electrophoresis
Part VIII. On the MG Values of Dyes

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MG values of dyes could be obtained in the horizontal-type apparatus permitting evaporation with 30% and 4% acetic acid solutions. In this experiment the fact that MG values are constant values not influenced by current, voltage, time of electrophoresis, dropped positions and length of filterpaper was both theoretically and experimentally proved, and conditions for regulating the applicable range of MG values were examined.

The authors reported that MG values of amino acids[1,2,3,4] and serum proteins[4,5] are constant regardless of current, voltage, time of electrophoresis, dropped position and length of filterpaper. In that occasion[3,4,5] it was found that for the establishment of MG value, a parallel relation between the graphs of dropped positions and migration distance of the migrants and the graph of dextran is required. However, when dyes were migrated in acetic acid solutions of 30% and 4%, the dyes which had parallel graphs with dextran were very small in number[6]. For this reason, further experiments of analysing these MG values in order to expand the application of these definitions were carried out.

EXPERIMENT AND DISCUSSIONS

I Materials and method Electrophoresis of 31 kinds of dyes was conducted with acetic acid solutions of 30% and 4% using the same apparatus as previously described in the experiment process[6].

II Mg values of dyes The graph in Fig. 1 showing the relation of dropped positions of migrants A, B, C and D with migration distance is not parallel with that of dextran but, a parallel relation is maintained only among A, B, C and D, one another. Suppose $V_0'{}_A$, $V_0'{}_B$, $V_0'{}_C$ and $V_0'{}_D$ represent the apparent initial velocity of A, B, C and D respectively, and the apparent migration distance i.e., MD'_A, MD'_B, MD'_C and MD'_D shown after the time-length of t, then as each straight-line graph being parallel to each other, migration distance may be calculated by the following formulae[3,4]:

$$\int_0^t V_0'{}_A\ (a'/a)^t dt = MD'_A \qquad (1\text{–}1)$$

$$\int_0^t V_0'{}_B\ (a'/a)^t dt = MD'_B \qquad (1\text{–}2)$$

$$\int_0^t V_0'{}_C\ (a'/a)^t dt = MD'_C \qquad (1\text{–}3)$$

$$\int_0^t V_0'{}_D\ (a'/a)^t dt = MD'_D \qquad (1\text{–}4)$$

Suppose MG_B and MG_C represent the MG value of B and C respectively, the following formulae[3,4] are obtained:

$$MG_B = \frac{MD'_B - MD'_A}{MD'_D - MD'_A} \qquad (2\text{–}1)$$

$$MG_C = \frac{MD'_C - MD'_A}{MD'_D - MD'_A} \qquad (2\text{–}2)$$

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1) K. Sakamoto and K. Takeoka, This Bulletin, **20**, 98 (1956).

2) K. Sakamoto and K. Takeoka, *J. Agr. Chem. Soc. Japan*, **30**, 463 (1956).

3) K. Sakamoto and K. Saito, This Bulletin, **22**, 55 (1958).

4) K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 279 (1958).

5) K. Sakamoto and K. Saito, *J. Agr. Chem. Soc. Japan*, **32**, 275 (1958).

6) K. Sakamoto, K. Saito and A. Nagata, This Bulletin, **23**, 89 (1959).

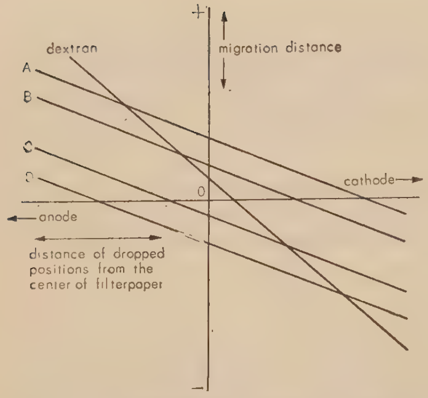
+
dextran
migration distance
A
B
C
D
cathode
0
anode
distance of dropped positions from the center of filterpaper
−

FIG. 1. The relationships between the migration distance of A (B, C, D and dextan) and dropped positions.

By substituting formulae (1–1), (1–2), (1–3) and (1–4) into the right-side of formulae (2–1), (2–2), $\mathrm{MG_B}$ and $\mathrm{MG_C}$ are represented by the following formulae:

$$\mathrm{MG_B}=\frac{V_{0\mathrm{B}}-V_{0\mathrm{A}}}{V_{0\mathrm{D}}-V_{0\mathrm{A}}} \qquad (3\text{–}1)$$

$$\mathrm{MG_C}=\frac{V_{0\mathrm{C}}-V_{0\mathrm{A}}}{V_{0\mathrm{D}}-V_{0\mathrm{A}}} \qquad (3\text{–}2)$$

Thus, MG values can be proved not to be influenced by the osmotic flow into filterpaper and the time of electrophoresis. That is, not to be affected by osmotic flow also means not to be influenced by dropped positions. Furthermore, when $V_{0\mathrm{A}}$, $V_{0\mathrm{B}}$, $V_{0\mathrm{C}}$ and $V_{0\mathrm{D}}$ respectively represent the net-initial velocity of A, B, C and D and V_{0el} represents the velocity of elecro-osmosis, formulae (3–1) and (3–2) are represented as:

$$\mathrm{MG_B}=\frac{V_{0\mathrm{B}}-V_{0el}-V_{0\mathrm{A}}+V_{0el}}{V_{\mathrm{cD}}-V_{0el}-V_{0\mathrm{A}}+V_{0el}}=\frac{V_{0\mathrm{B}}-V_{0\mathrm{A}}}{V_{0\mathrm{D}}-V_{0\mathrm{A}}} \qquad (4\text{–}1)$$

$$\mathrm{MG_C}=\frac{V_{0\mathrm{C}}-V_{0el}-V_{0\mathrm{A}}+V_{0el}}{V_{0\mathrm{D}}-V_{0el}-V_{0\mathrm{A}}+V_{0el}}=\frac{V_{0\mathrm{C}}-V_{0\mathrm{A}}}{V_{0\mathrm{D}}-V_{0\mathrm{A}}} \qquad (4\text{–}2)$$

Thus it can be observed that MG values are not under the influence of electro-osmosis. And as V_{0el} is the function of current, $V_{0\mathrm{A(B,C,D)}}$ having no relation with current, from formulae (4–1) and (4–2) it can be seen that the MG value is not under any influence of current. Furthermore, in formulae (4–1) and (4–2), $V_{0\mathrm{A}}$, $V_{0\mathrm{B}}$, $V_{0\mathrm{C}}$ and $V_{0\mathrm{D}}$ containing voltage, (v, in common), MG values being their quotients, are realized not to be under the influence of voltage. Consequently, as seen in Fig. 1, MG values are recognized to be constant values regardless of current, voltage, dropped positions and time of electrophoresis, provided the graph denoting the relation between dropped positions and migration distance among migrants is parallel to each other.

As previously reported[6,7], the dyes which have R values close to each other in adsorption analysis have almost parallel graphs representing the relation between dropped positions and migration distance, thus making it possible to obtain MG values of dyes belonging to groups A, B and C[6] with 30% acetic acid solution and those of groups B′, C′ and D′[6] with 4% acetic acid solution, respectively. The definitions of MG values of listed as follows:

1) 30% acetic acid solution

i) group A

$$\mathrm{MG}=\frac{\text{migration distance of another dye from malachite green}}{\text{migration distance of new coccine from malachite green}}$$

ii) group B

$$\mathrm{MG}=\frac{\text{migration distance of another dye from bismarck brown R}}{\text{migration distance of tartrazine from bismarck brown R}}$$

iii) group C

$$\mathrm{MG}=\frac{\text{migration distance of another dye from methylen blue}}{\text{migration distance of indigo carmine from methylen blue}}$$

2) 4% acetic acid solution

i) group B′

$$\mathrm{MG}=\frac{\text{migration distance of another dye from pontacyl green BL}}{\text{migration distance of new coccine from pontacyl green BL}}$$

ii) group C′

$$\mathrm{MG}=\frac{\text{migration distance of another dye from brom phenol blue}}{\text{migration distance of tartrazine from brom phenol blue}}$$

iii) group D′

$$\mathrm{MG}=\frac{\text{migration distance of another dye from malachite green}}{\text{migration distance of indigo carmine from malachite green}}$$

As for the dyes belonging to groups other than A, the MG values in average, of 30 examples as well as their standard deviations are given in Table I. These involve five different factors, i. e., current, voltage, time of electrophoresis, dropped positions and length of filterpaper, while the dropped quantity was fixed at 25 γ. The reason for fixing dropped quantity above the unit γ, is derived from the fact that variation of

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[7] K. Sakamoto and K. Saito., *J. Agr. Chem. Soc. Japan*, **32**, 390 (1958).

TABLE I. MG Values of Dyes

(I) A group a, b, c, d, e, f, g, h and i in the table is respectively quantitative combination of dyes in the following (γ):

	a	b	c	d	e	f	g	h	i
new coccine	25	100	100	12.5	12.5	100	100	12.5	12.5
test-dye	25	100	12.5	100	12.5	100	12.5	100	12.5
malachite geren	25	12.5	12.5	100	100	100	100	12.5	12.5

in 30% acetic acid solution,

(II) B group dropped quantity; 25γ, 0.20~0.38 mA/cm, 1~3 hours, in 30% acetic acid solution
(III) C group dropped quantity; 25γ, 0.19~0.38 mA/cm, 1~3 hours, in 30% acetic acid solution
(IV) B′ group dropped quantity: 25γ, 0 12~0.26 mA/cm, 40~120 minutes, in 4% acetic acid solution
(V) C′ group dropped quantity: 25γ, 0.12~0.29 mA/cm, 40~120 minutes, in 4% acetic acid solution
(VI) D′ group dropped quantity: 25γ, 0.12~0.27 mA/cm, 40~120 minutes, in 4% acetic acid solution
6.5~13 v/cm, dropped position: 8 cm toward the anode——8 cm toward the cathode from the center of filterpaper, length of filterpaper: 40 cm, 35 cm, Tôyô Filterpaper No. 50, in the horizontal-type apparatus permitting evaporation

(I)

new coccine	orange G	sunset yellow	wool violet 4 BN	pontacyl green BL	acid blue VS	rohda-mine B	mala-chite green	time (hour)	voltage (v/cm)	current (mA/cm)	dropped position	combi-nation of dyes	length of filter-paper (cm)
1.00	0.88	0.84	0.73	0.71	0.49	0.11	0	1	6.5	0.19	center	a	40
1.00	0.89	0.86	0.73	0.73	0.51	0.14	0	//	//	//	//	//	//
1.00	0.90	0.81	0.69	0.71	0.52	0.14	0	//	//	//	//	//	//
1.00	0.91	0.87	0.67	0.67	0.52	0.07	0	//	//	0.21	//	b	//
1.00	0.87	0.82	0.67	0.64	0.63	0.06	0	//	//	//	//	c	//
1.00	0.95	0.91	0.79	0.79	0.64	0.18	0	//	//	//	//	d	//
1.00	0.98	0.94	0.70	0.70	0.64	0.17	0	//	//	//	//	e	//
1.00	0.94	0.83	0.69	0.67	0.50	0.11	0	2	//	0.20	//	a	//
1.00	0.91	0.82	0.71	0.67	0.49	0.10	0	//	//	//	//	//	//
1.00	0.79	0.74	0.63	0.62	0.51	0.11	0	//	//	0.22	//	b	//
1.00	0.90	0.85	0.69	0.64	0.56	0.14	0	//	//	//	//	g	//
1.00	0.90	0.85	0.68	0.65	0.55	0.10	0	//	//	//	//	h	//
1.00	0.92	0.86	0.65	0.61	0.52	0.09	0	//	//	//	//	i	//
1.00	0.92	0.86	0.70	0.64	0.56	0.09	0	1	13.0	0.40	center	a	40
1.00	0.92	0.87	0.70	0.64	0.53	0.09	0	//	//	//	//	//	//
1.00	0.94	0.86	0.70	0.64	0.52	0.09	0	//	//	//	//	//	//
1.00	0.91	0.86	0.71	0.65	0.54	0.10	0	//	//	//	//	//	//
1.00	0.93	0.87	0.70	0.67	0.53	0.10	0	//	//	//	//	//	//
1.00	0.90	0.83	0.69	0.61	0.56	0.20	0	//	//	//	//	b	//
1.00	0.93	0.84	0.73	0.68	0.54	0.14	0	//	//	//	//	c	//
1.00	0.98	0.94	0.83	0.78	0.70	0.15	0	//	//	//	//	d	//
1.00	0.99	0.93	0.81	0.76	0.64	0.14	0	//	//	//	//	e	//
1.00	0.87	0.79	0.69	0.68	0.58	0.18	0	//	//	//	//	f	//
1.00	0.93	0.82	0.72	0.65	0.54	0.17	0	//	//	0.42	//	g	//
1.00	0.89	0.78	0.67	0.64	0.55	0.13	0	//	//	//	//	h	//
1.00	0.91	0.84	0.71	0.65	0.52	0.19	0	//	//	//	//	i	//

new coccine	orange G	sunset yellow	wool violet 4 BN	pontacyl green BL	acid blue VS	rohda-mine B	mala-chite green	time (hour)	voltage (v/cm)	current (mA/cm)	dropped position	combi-nation of dyes	length of filter-paper (cm)
1.00	0.90	0.83	0.68	0.62	0.49	0.08	0	2.5	6.5	0.21	8cm anodic side from the center	a	40
1.00	0.92	0.82	0.68	0.61	0.56	0.11	0	//	//	//	//	//	//
1.00	0.90	0.84	0.66	0.68	0.46	0.11	0	//	//	//	//	//	//
1.00	0.94	0.89	0.73	0.69	0.60	0.10	0	//	//	//	8cm cathodic side from the center	//	//
1.00	0.93	0.85	0.73	0.69	0.61	0.14	0	//	//	//	//	//	//
1.00	0.89	0.79	0.66	0.60	0.50	0.15	0	//	//	//	8cm anodic side from the center	b	//
1.00	0.91	0.85	0.64	0.57	0.44	0.07	0	//	//	//	//	c	//
1.00	0.88	0.82	0.67	0.59	0.53	0.18	0	//	//	//	//	d	//
1.00	0.97	0.89	0.77	0.69	0.56	0.19	0	//	//	//	//	e	//
1.00	0.93	0.85	0.77	0.71	0.62	0.08	0	//	//	//	8cm cathodic side from the center	f	//
1.00	0.95	0.86	0.77	0.68	0.61	0.07	0	//	//	//	//	g	//
1.00	0.92	0.84	0.83	0.65	0.57	0.09	0	//	//	//	//	h	//
1.00	0.96	0.87	0.69	0.65	0.57	0.08	0	//	//	//	//	i	//
1.00	0.92	0.85	0.69	0.63	0.50	0.10	0	3	6.5	0.20	center	a	35
1.00	0.91	0.85	0.68	0.63	0.51	0.10	0	//	//	//	//	//	//
1.00	0.93	0.80	0.70	0.64	0.50	0.09	0	//	//	//	//	//	//
1.00	0.91	0.85	0.68	0.64	0.51	0.14	0	//	//	//	//	//	//
1.00	0.92	0.85	0.70	0.64	0.51	0.11	0	//	//	//	//	//	//
1.00	0.94	0.85	0.73	0.66	0.54	0.08	0	//	//	0.22	//	b	//
1.00	0.91	0.85	0.68	0.60	0.49	0.06	0	//	//	//	//	c	//
1.00	0.96	0.87	0.74	0.69	0.56	0.11	0	//	//	//	//	d	//
1.00	0.95	0.88	0.71	0.65	0.55	0.25	0	//	//	//	//	e	//
1.00	0.96	0.87	0.74	0.70	0.56	0.23	0	//	//	//	//	f	//
1.00	0.89	0.83	0.64	0.60	0.50	0.11	0	//	//	0.23	//	g	//
1.00	0.91	0.85	0.71	0.67	0.56	0.10	0	//	//	//	//	h	//
1.00	0.93	0.86	0.67	0.61	0.51	0.09	0	//	//	//	//	i	//
1.00	0.88	0.82	0.67	0.61	0.50	0.09	0	3	6.5	0.19	center	a	40
1.00	0.89	0.83	0.68	0.62	0.51	0.10	0	//	//	0.19	//	//	//
1.00	0.90	0.84	0.69	0.63	0.52	0.11	0	//	//	0.21	//	//	//
1.00	0.91	0.85	0.70	0.64	0.53	0.12	0	//	//	0.22	//	//	//
1.00	0.92	0.86	0.71	0.65	0.54	0.13	0	//	//	0.22	//	//	//
1.00	0.85	0.79	0.65	0.61	0.52	0.09	0	//	//	0.19	//	b	//
1.00	0.92	0.85	0.69	0.63	0.52	0.08	0	//	//	0.20	//	c	//
1.00	0.96	0.90	0.78	0.73	0.62	0.14	0	//	//	0.20	//	d	//
1.00	0.98	0.92	0.76	0.72	0.61	0.16	0	//	//	0.21	//	e	//
1.00	0.85	0.78	0.67	0.63	0.53	0.13	0	//	//	0.21	//	f	//
1.00	0.92	0.85	0.68	0.63	0.53	0.09	0	//	//	0.22	//	g	//
1.00	0.91	0.81	0.67	0.62	0.53	0.09	0	//	//	0.22	//	h	//
1.00	0.94	0.86	0.66	0.62	0.51	0.09	0	//	//	0.23	//	i	//
1.00	0.92 ±0.03	0.85 ±0.04	0.70 ±0.04	0.66 ±0.04	0.54 ±0.04	0.13 ±0.04	0 0						

(II)

	tartrazine	amaranth	naphthol yellow S	alizarine red S	brom phenol blue	methyl orange	crystal violet	fuchsine	bismarck brown R
MG	1.00	0.94±0.03	0.87±0.03	0.75±0.03	0.68±0.03	0.46±0.04	0.20±0.04	0.12±00.3	0

(III)

	indigo carmine	orange II	uranine	methyl violet	auramine	methylen blue
MG	1.00	0.82±0.03	0.40±0.03	0.23±0.04	0.11±0.04	0

(IV)

	new coccine	orange G	naphthol yellow S	sunset yellow	pontacyl green BL
MG	1.00	0.72±0.04	0.65±0.04	0.42±0.05	0

(V)

	tartrazine	amaranth	kiton blue A	brom phenol blue
MG	1.00	0.79±0.05	0.17±0.03	0

(VI)

	indigo carmine	alizarine red S	methyl orange	malachite green
MG	1.00	0.84±0.02	0.52±0.05	0

concentration and dropped volume are directly represented in different angles formed between the graph representing the relation of migration distance with dropped positions of the dyes and abcissa. As for the dyes belonging to group A MG values were obtained in consideration of concentration and dropped volume in adsorption analysis, the approvable variation of dropped quantity being within the range of 12.5~100γ.

As it is seen from this table the MG value was in accordance with the above definition the constant values without being affected by any of the above electrophoresis conditions. However, these MG values in comparison with those values of amino acids[3,4] and serum proteins[3,5], involve a somewhat expanded range of error. As the MG value is derived from the condition of the parallel relation indicated in the graph between migration distance and dropped positions of each migrant, deviation of the values is probably due to the fact that the dyes classified in each group, strictly speaking, do not bear parallel graphs. Among the dyes of group A[6], the MG of kiton blue A could not be obtained as its R value was liable to receive the influence of dropped quantity. In regard of sample solutions containing dyes which carry different MG values when dropped quantity either increases or decreases, it is difficult to identify the dyes contained by means of MG value. In consideration of MG values of dyes belonging to group A, the MG is regarded to be applicable to a substance with a R value of 1.00 in adsorption analysis, within the range where the R value remains at 1.00.

SUMMARY

MG values of dyes classified in groups by means of adsorption analysis with acetic acid solutions of 30% and 4% could be obtained in a horizontal type apparatus permitting evaporation. After conducting analysis of MG values under the new definition, and by experimental measurement, they were found to be constant values not affected by conditions of electrophoresis.

Acknowledgement In concluding this paper, the authors wish to express their gratitude to Prof. Y. Ōshima of Kyushu University for his generous guidance. Thanks are due to San-ei Chemical Manufacturing Co., Ltd. for kindly supplying the dyes used in this work.

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Studies on the Activities of Bacteria in Soy Sauce Brewing

Part V. The Effects of *Aspergillus sojae*, *Pediococcus soyae*, *Bacillus subtilis* and *Saccharomyces rouxii* in Purely Cultured Soy Sauce Brewing

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The soy koji mold *Aspergillus sojae* is purely cultured on the materials of soy koji, and soaked in the sterile concentrated sodium chloride solution (26%). To it, *Ped. soyae* and *Sacch. rouxii* are aseptically inoculated and their roles playing in soy sauce brewing are examined. *Bacillus subtilis* is added in the form of wheat bran culture into the purely cultured soy mash and its influencies are observed.

The appropriate addition of the bran culture of *Bac. subtilis* favors for the development of necessary aroma, and eliminates the polypeptide-like turbidity in soy sauce. The growth of *Ped. soyae* in soy mash stimulates the formation of organic acids, and makes the mash acidic which is necessary for the sound fermentation, removes undesirable flavors, and adds the indispensable good flavors for soy sauce. But the rapid lowering of pH results in the depression of *Aspergillus* Alkaline Proteinase activities[1,2,3], followed by its inactivation, thus the percentage of solubilization of the protein material decreases. The colour of soy sauce also becomes lighter. *Sacch. rouxii* produces ethanol, and cooperating with *Ped. soyae*, plays an important part for developing the soy sauce flavor. *Asp. sojae* is the most important microorganism for soy sauce brewing. Almost all the essential enzymes for soy sauce brewing originates in *Asp. sojae*, as a result, fundamental chemical components, aroma and flavor are developed by enzymes from this mold. The soy sauce brewed by *Asp. sojae* alone, however, is not equal to the one which is made with mixed organisms, but it is only the mother body of soy sauce. The solubilization ratio of the protein material has also reached to the maximum without the aid of other microorganisms. However, the production of organic acids and ethanol is insufficient, polypeptide-like turbidity in soy sauce has remained, and aroma and flavor are not as well as the normally brewed soy sauce.

By addition of bacterial and yeast cultures, attempts for improving the quality of soy sauce were frequently reported. But they were all dealing with open natural cultures, and no proper roles of individual microorganisms were extensively studied. In this experiment, the important four microorganisms relating to the soy sauce brewing were all purely cultured and added, and their roles were elucidated by chemical analysis, by enzyme activities and by sensuous tasting. The four important microorganisms dealt with were; *Aspergillus sojae*[4,5], *Pediococcus soyae*[6], *Bacillus subtilis*[1,7,8] and *Saccharomyces rouxii*[9]. The other isolated microorganisms relating to soy suace brewing;

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[1] K. Sakaguchi, This Bulletin, **22**, 345 (1958).

[2] T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1957).

[3] W. G. Crewther and F. G. Lennox, *Aust. J. Biol. Sci.*, **6**, 428 (1953).

[4] K. Sakaguchi and K. Yamada, *J. Agr. Chem. Soc. Japan*, **20**, 64, 141 (1944).

[5] K. Yamamoto, This Bulletin, **21**, 308, 313, 319 (1957)

[6] K. Sakaguchi, This Bulletin, **22**, 353 (1958); **23**, 22 (1959).

[7] Y. Ishimaru, *J. Agr. Chem. Soc. Japan*, **9**, 859, 953, 1143 (1933).

[8] K. Matsumoto, *J. Soc. Brewing Japan*, **21** (1926), **22** (1927), **23** (1928), **24** (1929).

[9] H. Onishi, This Bulletin, **21**, 137, 143, 151 (1957).

namely lactic acid bacteria growing in soy koji[6], *Brettanomyces*[7,8] and *Torulopsis*[9,10,11] appearing in the late stage of soy mash fermentation, were excluded in this study because of their meager influences on the brewing.

EXPERIMENTAL

Preparation of purely cultured soy mash. A large two necked Komagome type flask (Fig. 1) was used for the pure culture of soy koji. To it, 49.5 g of defatted granular soy bean, 24.2 g of parched and cracked wheat grain and 7.9 g of wheat bran were added and autoclaved for forty minutes at 1.5 atm/cm². The defatted soy bean and wheat bran were pre-treated by spraying with boiling water. The sterilized materials were inoculated with *Aspergillus sojae* spores and incubated for three days at 30°C. After the whole materials were covered with mycelia and spores, the soy koji was aseptically transfered to the bottle illustrated in Fig. 2, and soaked with 180 ml of autoclaved brine containing 26.00% sodium chloride. The concentration of salt was too high in this case, and it should be corrected to about 24%. The salt used was the Arabian bay salt, and its sodium chloride content was about 91%. After the soaking, the bacterial contamination was inspected using eurocidin media[12]. The bacteria-free bottles were incubated at 30°C for two and half months. Time to time the air was bubbled through the longer cotten tube into the bottle to stimulate the growth of yeast which was inoculated latter.

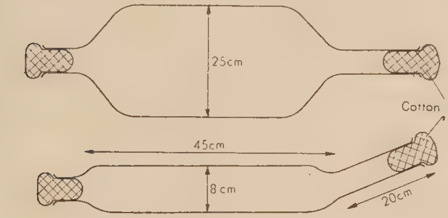

FIG. 1. The Two Necked Komagome Type Flask for the Cultivation of Purely Cultured Soy Koji.

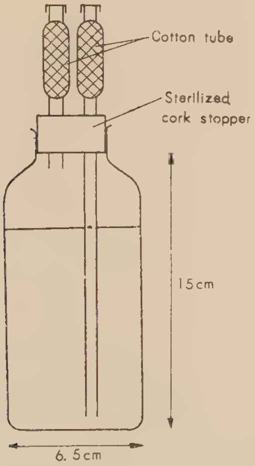

FIG. 2. The Bottle for the Brewing of Purely Cultured Soy Mash.

Inoculation of *Pediococcus soyae*. *Ped. soyae dl* 2[6] was subcultured in 5 ml of the broth, containing yeast extract (Difco) 0.3%, polypepton (Takeda) 0.5%, glucose 1.0%, dipotassium phosphate 0.5% and sodium chloride 7.0%. This culture was incubated anaerobically from three to six days at 30°C, then poured into the purely cultured soy mash three days after the soaking of soy koji.

Inoculation of *Saccharomyces rouxii*. *Sacch. rouxii* strain No. 7[13,9] isolated from the soy mash was subcultured in 5 ml of rice koji water (Bllg 10°) containing 7% sodium chloride for three or six days at 30°C. This culture was inoculated into the purely cultured soy mash on the same day as *Ped. soyae*.

Addition of bran cultured *Bacillus subtilis*. Five grams of wheat bran, 1 g of soy bean powder and 5 ml of water in 150 ml Fernbach flask was autoclaved at 1.5 atm/cm² for forty minutes. Strong proteinase producers, *Bac. subtilis var. Biotecus* A-538 (Nagase Industries Co.,) *Bac. subtilis* strain Fn and H-127-H (Institute of Applied Microbiology, University of Tokyo) were inoculated and cultured respectively for three days at 30°C. The resulted each bacterial bran was added into the purely cultured soy mash at the time of soaking with brine.

Mixed culture of *Asp. sojae* and *Bac. subtilis var. Biotecus* A-538. *Asp. sojae* was inoculated on the autoclaved soy koji materials, and after one day's culture, *Bac. subtilis var. Biotecus* A-538 was inoculated. The incubation was continued for two days thereafter.

Brewing of the factory soy koji. The soy koji was supplied from the Seventh Factory, Noda Shoyu Co.. Four hundred grams of the soy koji was soaked

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10) H. Onishi, read on the General Meeting of the Soc. Agr. Chem. Japan on April, 1957.

11) M. Mogi and H. Onishi, *J. Ferment. Technol.*, **33**, 396 (1955).

12) K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **28**, 758 (1954), *Rep. Noda Inst. Sci. Res.*, **2**, 25 (1958).

13) H. Onishi, unpublished.

with 700 ml of crude concentrated brine containing 22.60% sodium chloride. The mash was incubated for three months at 30°C in the same manner as the purely cultured soy mash.

Measurement of hydrogen ion concentration. The pH Test Papers (Toyo Roshi Co.), Brom Cresol Purple and Brom Cresol Green were calibrated with a Beckman pH meter and were used.

Titration of total acid. One millilitre of soy sauce was diluted to 50 ml with distilled water, and titrated with N/20 sodium hydroxide to pH 7.0 using a Beckman pH meter Model G. The value was expressed by milligram of lactic acid per ml of soy sauce[14].

Measurement of proteinase activity. The method described in the previous paper[1] was followed.

Assay of free amino nitrogen[14,15]. Formol nitrogen was titrated potentiometrically according to the method of Sakasai and Yokotsuka.

Assay of ammonium. The soy sauce was diluted 50 fold, and Conway's method of microdiffusion analysis[16] was applied.

Assay of polypeptide[17]. To 10 ml of soy sauce, 25 ml of dist. water, 5 ml of 10% sodium tungstate solution and 10 ml of 1 N sulfuric acid were added successively and polypeptides in soy sauce were precipitated in a refrigerator over night. The precipitate was collected by centrifugation, washed with 10% tungstic acid, and the nitrogen content of the material was measured by Kjeldahl's method.

Assay of glutamic acid. The soy sauce was diluted 1000 fold, and glutamic acid content was bioassayed using *Lact. arabinosus* as test organism[18,19].

Measurement of α-amylase activity. The unpasteurized soy sauce was diluted ten fold with M/15 phosphate buffer pH 6.0, and used as an enzyme solution. Two millilitre of the enzyme solution was poured into 10 ml of 0.4% soluble starch solution (pH 6.0) and incubated at 30°C. A half millilitre of the mixed solution was taken out at five minutes intervals, and poured into 2 ml of the 2 N I_2 solution. The time when the blue colour of starch-I_2 reaction has disappeared, was recorded.

Assay of reducing sugars. Fehling-Lehmann-Schoorl's method was followed.

Colour intensity. The soy sauce was diluted 12 fold, and measured by an electrophotometer (Erma) with the transmission light through 420 mμ filter. The value was given by the optical density.

RESULTS AND DISCUSSION

As shown in the Table, 10 lots and 18 bottles of purely cultured soy sauces were prepared and brewed for two and half months at 30°C, and their enzymic activities, chemical compositions, solubilization ratios of materials, flavors and aromas were examined. Since all microorganisms added were purely cultured, the roles of each of them were clearly shown in this table. The tested organisms were; *Asp. sojae strain KS.*[4,5], three strains of strong protease producers *Bacillus subtilis var. Biotecus* A-538 (Nagase Industries Co.), *Bac. subtilis* strain Fn and H-127-H (Institute of Applied Microbiology, University of Tokyo), *Pediococcus soyae*[6] strain *dl* 2 and *Saccharomyces rouxii* No. 7[9,10,11].

Asp. sojae is the so called soy koji mold, and it is the most important microorganism for soy sauce making. The organism produces proteases, amylases, other important enzymes for soy sauce brewing, and also aromas and flavors. *Bacillus subtilis* grows only in the koji making stage. *Pediococcus soyae* is the only active bacteria in soy mash, and produces lactic acid. *Saccharomyces rouxii* also grows well in soy mash and ferments only glucose but no other sugars.

The combinations of microorganisms were; *Asp. sojae* koji alone, *Asp. sojae* koji plus *Bac. subtilis* brans, mix-cultured koji of *Asp. sojae* and *Bac. subtilis*, *Asp. sojae* mash plus *Ped. soyae* culture, *Asp. sojae* mash plus *Sacch. rouxii* culture, *Asp. sojae* mash plus *Sacch. rouxii* and *Ped. soyae* culture, *Asp. sojae* koji plus *Bac. sub. var. Biotecus* bran plus *Sacch. rouxii* culture plus *Ped. soyae* culture, and open cultured factory soy koji.

The tasting of the brewed soy sauce were entrusted to four members of tasting committee in Noda Shoyu Co.. The hydrogen ion con-

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14) T. Sakasai and T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **32**, 144 (1958)

15) M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 395, 691 (1936).

16) E. J. Conway, Microdiffusion Analysis and Volumetric Error, p. 75 (1950).

17) S. Hori et al., *J. Agr. Chem. Soc. Japan*, **30**, 515 (1952).

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centration of the soy mash was measured on seventh, thirteenth day, and two and half months after the soaking of soy koji. The proteinase activity of the soy mash juice was measured at three pH values, 3.5, 7.0 and 10.0 on the 7th day after the soaking, and two and half months later, at pH 7.0.

On the samples brewed for two and half months, the other properties of the products were examined, because, the fermentation and maturing were usually concluded in three months when the soy sauce brewing was conducted in the warmed chamber at 30°C, and the product was almost identical with the normal soy sauce brewed for a year under uncontrolled temperature.

Roles played by *Asp. sojae.* The soy koji mold *Asp. sojae* was undoubtedly the most important organism for soy sauce brewing. By its biochemical actions during koji making stage, and by its enzymatic actions in soy mash, the mother body of the soy sauce was produced. As shown on the table, the percentage of solubilized proteins (mainly soy bean protein) into the soy sauce (The value is called as utilizing ratio of total nitrogen.) reached to the maximum without the aid of other microorganisms. The proteinase activity of fresh soy mash juice also did not fall below other mixed cultured soy mashes. This provides an evidence for the previous report[1)] that the proteinases in soy mash are almost *Aspesgillus* origin.

Because of the lack of lactic acid bacteria, the pH of the mash was not rapidly lowered, and the proteinase activity was not so decreased even after two and half months incubation at 30°C.

The liberation of glutamic acid, α-amylase activity and solubilization ratio of starch in raw material were also normal.

It is apparent, however, that *Asp. sojae* is able to make *only the mother body* of soy sauce, and it is not the soy sauce in itself. The absence of lactic acid bacteria caused the acid insufficiency, and the pH was not lowered below pH 5.5 even after two and half months incubation at 30°C. If the mash is open cultured, it will be easily rotten by salt tolerant *Bacilli*[7,8)], which is suppressed by the low pH value in the normal soy mash[6)].

Although it was not a bad smell, soy sauce which was made with *Asp. sojae* alone had a different aroma and flavor from the normal one. It has a specific drug like odour, which was removed by the growth of *Ped. soyae* and *Sacch. rouxii.*

The absence of yeast resulted in the deficiency of ethanol, normal content of which was from 2.0% to 3.0% in the case of unpasteurized soy sauce.

A distinct fault of the purely cultured soy sauce which was made with *Asp. sojae* alone was the appearance of a large amount of polypeptide-like white precipitate in soy sauce. The precipitate appeared in soy sauce when it was refrigerated, heated, or even diluted by water. It is a troublesome character for an article of commerce.

Roles played by *Bacillus subtilis.* *Bac. subtilis* and other aerobic spore forming bacteria grow only in soy koji making stage, and their spores are surviving in soy mash[1)]. These bacteria were formerly considered as a harmful contaminant in soy koji, but this experiment proved that the appropriate addition of such *Bacilli* which does not produce unfavorable flavor, was useful and even necessary for soy sauce brewing. A specific drug-like odour of soy sauce brewed with *Asp. sojae* was diminished by the addition of bran culture of *Bac. subtilis*, further, the polypeptide-like turbidity of soy sauce also disappeared.

This improvement of soy sauce flavour is no wonder because there exists usually 10^6–10^8 cells of *Bacilli* per gram of the open cultured normal soy koji. But both mixed culturing of *Bac. subtilis var. Biotecus* A-538 with soy koji and the addition of its bacterial bran, gave soy sauce the so-called *Bacillus* odour. Therefore it is necessary to select strains and to regulate the excessive growth of *Bacilli.*

ANALITICAL AND TASTING DATA OF

Related microorganisms	NaCl concentration %	pH after			Total acid as lactic acid mg/ml	Proteinase activity				Total nitrogen in soy sauce 10mg/ml, %	Utilizing ratio of total nitrogen %	Amino N / Total N %
		7 days	13 days	2.5 months		after 7 days pH 3.5	pH 7.0	pH 10.0	2.5 Months pH 7.0			
Asp. sojae.	20.34	6.6	7.0	5.5	7.4	0.1	4.5	5.1	4.0	1.53	77.7	56.9
	20.27	6.4	6.6	5.3	7.9	0.3	4.1	5.2	3.5	1.50	76.3	56.0
	20.43	6.6	6.4	5.3	7.2	0.1	3.5	3.9	3.1	1.47	74.5	55.6
Asp. sojae, Bac. sub. var. Biotecus.	20.36	6.6	6.4	5.3	6.4	0.0	5.2	3.8	5.0	1.54	77.8	56.6
	20.50	6.4	6.4	5.5	5.0	0.1	3.9	4.7	4.3	1.47	73.7	58.6
Asp. sojae, Bac. sub. Fn	20.30	6.6	7.0	5.7	4.1	0.1	4.8	3.9	2.8	1.50	76.0	57.5
	20.36	6.8	6.8	5.3	5.4	0.1	4.8	3.1	3.2	1.51	76.6	55.6
Asp. sojae, Bac. sub. H-127-H	20.59	6.8	6.8	5.5	4.5	0.0	2.6	2.6	2.3	1.46	73.1	57.3
	20.41	6.6	6.8	5.7	3.9	0.0	3.1	2.9	3.4	1.45	73.3	56.2
Asp. sojae, Bac. sub. var. Biotecus. (Mixed culture)	20.54	6.9	7.2	5.3	6.3	0.2	4.8	4.0	3.3	1.50	75.6	57.6
	20.46	6.9	7.2	5.3	7.2	0.6	5.6	4.2	4.2	1.54	77.5	54.7
Asp. sojae, Sacch. rouxii No. 7.	20.62	6.8	6.8	5.5	4.7	0.0	3.3	3.7	3.0	1.41	70.3	58.2
	20.52	6.8	7.0	5.5	5.0	0.1	3.4	3.5	3.0	1.46	73.5	57.5
Asp. sojae, Ped. soyae dl 2	20.10	6.4	4.9	4.7	11.7	0.0	3.6	5.1	0.6	1.30	66.5	58.4
	20.35	6.6	4.9	4.5	11.2	0.1	3.0	3.8	0.5	1.18	59.8	61.0
Asp. sojae, Ped. soyae dl 2, *Sacch. rouxii* No. 7.	20.50	6.4	5.6	4.7	10.5	0.2	2.9	3.4	0.5	1.30	65.3	54.9
Asp. sojae, Bac. sub. var. Biotecus, Ped. soyae, Sacch. rouxii	20.33	6.6	4.9	4.7	10.9	0.0	6.1	4.6	1.3	1.40	71.0	58.7
Factory soy koji.	17.78	6.0	5.6	4.5	12.2	2.0	6.3	5.4	1.7	1.37	——	58.7

By addition of *Bacillus subtilis*, no increase of proteinase activity was observed, and the solubilization ratio of protein in raw material (represented by the utilizing ratio of total nitrogen) also did not increase. But the appearance of protein-like turbidity in soy sauce was effectively inhibited by addition of *Bacillus subtilis*. This phenomenon probably is due to the breaking of certain peptide bonds which is resistant to the attack of *Aspergillus* proteinases, so even the small quantity of different *Bacillus* proteinase plays an important role.

The α-amylase activity was increased by the addition of this bacteria. The utilizing ratio of starch was, however, not improved.

Roles played by *Pediococcus soyae*. In this experimental brewing, the salt concentration of purely cultured soy mash was too high by accident, 2 or 3% higher than the normal concentrations. Even in these high salt concentrations at 20.2–20.6%, *Ped. soyae* grew well and the mash was rapidly acidified to pH 4.9 within ten days after the inoculation. The hydrogen ion concentration of the other lots were pH 7.0–6.4 on the same day, and no acid production was observed by other organisms. Their final hydrogen ion concentration were pH 5.3–5.5, and have not reached to the normal pH value of 4.8–4.5 without the aid of *Ped. soyae*. The total acidity was also paralleled

Purely Cultured Soy Sauces

NH_3-N / Total N %	Turbidity in Soy Sauce	Polypeptide N[17] / Total N %	Glutamic acid-N / Total N %	α-amylase activity ($Starch-I_2$) min.	Reducing sugars %	Alcohol %	Utilizing ratio of starch %	Color (420 mμ) Optical Density	Tasting
19.0	+	13.4	8.50	35	3.41	0.28	27.2	0.98	Mother body of soy sauce flavor, but not the soy sauce flavor itself. Lacked in acid taste. Special drug-like odour as phenol or related substances.
16.4	+	10.5	8.33	38	3.01	0.12	22.3	0.92	
18.3	++	11.3	8.16	35	2.97	0.13	17.7	0.89	
23.5	−	11.3	8.44	25	2.58	0.10	18.3	1.03	Drug-like odour was diminished. Moderate flavor. A necessary flavor for the soy sauce was developed. Lacked in acid taste.
24.3	−	11.4	8.84	25	3.61	0.13	26.3	0.96	
19.7	−	12.4	8.60	33	3.14	0.17	23.9	0.88	
19.5	−	10.1	9.59	34	2.68	0.15	20.4	0.95	
19.0	−	11.0	8.84	34	2.99	0.21	23.1	0.93	
19.2	−	11.0	8.69	34	3.08	0.23	24.1	0.83	
19.5	−	11.6	8.48	<10	2.67	0.07	19.0	1.01	Bacillus odour.
18.3	±	11.5	8.18	<10	2.61	0.07	18.8	1.02	
19.4	±	11.4	8.58	35	1.97	0.61	21.5	0.82	Too strong flavor of yeast fermentation, flut this flavor might be necessary for the soy sauce.
18.7	±	12.0	8.36	40	1.86	0.64	21.3	0.82	
23.4	++	11.5	8.00	90	2.28	0.15	17.9	0.66	Odd smells were almost removed. The essential flavor for the soy sauce was developed.
25.3	++	11.6	8.14	110	2.44	0.07	17.7	0.70	
22.2	++	13.3	7.69	120	1.78	0.28	15.9	0.66	Smells almost identical with soy sauce.
23.7	++	10.9	7.71	70	1.67	0.25	14.9	0.72	Bacillus odour prevailed.
11.2	−	5.2	9.56	45	5.18	0.02	——	0.73	Yeast fermentation was insufficient.

with the hydrogen ion concentration.

One of the most important characteristics of this lactic acid bacterium is the ability to form essential aromas and flavors for soy sauce. Its growth clearly eliminated the drug-like smell which was pronounced in the purely cultured soy sauce of *Asp. sojae*, and aromas became mild and well-matured. This activity of improving soy sauce aroma and flavor was so distinguished that the necessity of *Ped. soyae* for soy sauce brewing was indisputable despite some disadvantages of this organism described later.

It was mentioned that the growth of this microorganism lowered pH of the mash below pH 5.0, and protect the soy mash from spoilage by salt tolerant *Bacilli*. Another important effect of low pH value is to stimulate the growth of salt tolerant yeasts. Onishi[9] reported that, in 18% salted media, *Sacch. rouxii* could grow only in the range of pH 5.0–4.0. (The value was measured by the pH Test Pater (Toyo Roshi Co.). Brom Cresol Green. When it was measured by a Beckman pH meter Model G, about 0.6 higher pH values were obtained in the case of highly salted solutions.) Without the growth of *Ped. soyae*, the growth and fermentation of yeasts is imperfect.

As stated above, *Ped. soyae* is indispensable for the soy sauce brewing, but its too early growth in soy mash decreases the yield of soluble nitrogen in soy sauce. This microorganism has only the weak proteinase and peptidase in the cell, but had neither extracellular proteolytic enzymes nor the co-operating ability with *Aspergillus* proteinases[6]. On the other hand, the rapid growth of the lactic acid bacteria acidifies soy mash, and the optimal hydrogen ion concentration of *Aspergillus* alkaline proteinase[1,2,3,5,20] (pH 6.5–11.0) was soon missed. The proteinase activity of soy mash at pH 5.0 is about one tenth of that at pH 7.0. Moreover, the *Aspergillus* alkaline proteinase is rapidly inactivated because of the low pH of mash. The table shows that about eight tenth of the proteinase activity at pH 7.0 remained after two and half months brewing at 30°C in *Ped. soyae* omitted lots, while the lots with *Ped. soyae* kept only about two tenth of the activity. Accordingly, about 10% decrease of the solubilization ratio of total nitrogen in raw material was recognized on the lots.

The inactivation of *Asp. sojae* proteinases also caused the marked increase of the polypeptide-like precipitation in soy sauce, which was probably due to the imperfect digestion of protein.

The other actions played by *Ped. soyae* were; the increase of liberated ammonium, the inactivation of α-amylase of *Asp. sojae*, the decrease of the utilizing ratio of starch in raw material.

The colour intensity of soy sauce was markedly decreased by this organism, reaching to the light colour of the unpasteurized normal soy sauce.

In general, *Ped. soyae* is quite indispensable for forming the soy sauce aroma and flavour, and for conducting the sound brewing process in the open culture, but its acid production caused the inactivation of various enzymes of *Asp. sojae*. In order to overcome these unfavorable points, it is desirable to suppress the growth of *Ped. soyae* in the early stage of brewing, and after the enzymes of *Asp. sojae* have played larger parts of their roles, namely two or three months later, *Ped. soyae* should grow vigorously to acidify the soy mash and to develop the good flavor to soy sauce. From this view, it is reasonable that when soy koji is prepared and soaked in the brine during winter time, the resulted soy mash is superior both in its solubilization ratio of materials and in its quality.

Roles played by *Saccharomyces rouxii*. The salt tolerant yeast *Saccharomyces rouxii* strain No. 7[9,10,11], the former name of which was *Zygosaccharomyces major*, grows well in soy mash if it is sufficiently aerated. Although the yeast did not grow satisfactorily because of the insufficient aeration in this experiment, the effects of yeast growth is apparently distinguishable on the table.

The inoculation of the yeast resulted in the consumption of reducing sugars and the production of ethanol. A specific fermentation odour of yeast was not a bad smell in itself, but in this experiment, it was too intense and unfamiliar for soy sauce. The mixed inoculation of both *Ped. soyae* and *Sacch. rouxii*, however, have formed the most satisfactory aroma and flavour almost approximating to those of the normal soy sauce. That is, the yeast fermentation alone can not form the good flavor in soy mash, but the cooperation with *Ped. soyae* is quite necessary.

No other eminent activities of the yeast were observed in this experiment. Acids were not formed, and no influences on *Asp. sojae* enzymes were observed. The decolorizing activity for soy mash exists, but not so strong as that of *Ped. soyae*.

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Effect of Ultraviolet Light Irradiation on the Peptide Bonds of Protein

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In order to examine whether the peptide bonds of proteins are hydrolyzed with the ultraviolet light irradiation or not, egg white lysozyme, α-casein and β-lactoglobulin solutions were irradiated and the chemical analysis of the amino groups, total acidic and basic groups of each protein was carried out. As the result no significant change in the amount of these groups occurred before and after the irradiation. It is concluded that the peptide bonds of protein are not hydrolyzed by the irradiation and decomposition of tryptophan as reported in the previous paper[1] might occur without peptide linkage cleavage.

It has long been in question whether the peptide bonds of proteins are hydrolyzed by the ultraviolet light irradiation, or not. Several investigators assumed that one of the primary actions of ultraviolet light on proteins was to hydrolyze the peptide bonds adjacent to the UV chromophores tyrosine, tryptophan and phenylalanine residues in the protein molecule.

Since these evidences were all indirect, present investigation was designated to reexamine this problem more adequately by performing chemical determination of amino and carboxyl groups after the irradiation of ultraviolet light to cause possible physical and chemical changes in the proteins.

EXPERIMENTAL AND RESULTS

Materials and Methods. Egg white lysozyme was prepared by the procedure of G. Alderton[2], and recrystallized three times and lyophilized. α-Casein and crystalline β-lactoglobulin were given by S. Sakamura in this laboratory.

Each protein was dissolved in water and pH was adjusted to the range of 6.0 to 7.0, or dissolved in M/15 phosphate buffer solution (pH 6.0–7.0). In the case of basic group determination, M/10 citrate buffer solution (pH 2.2) was used. The concentrations of protein were 1 or 0.5 percent. The solutions were put into the glass vessel (3 cm diameter × 5 cm) covered with quartz glass and irradiated with a 200 watt mercury lamp at the distance of 15 cm for fifteen to twenty hours. The temperature of the solutions did not rise above 15°C during the irradiation.

Determination of Acidic and Amino Groups. The Søensen formol titration method as modified by J. H. Northrop[3] was used to estimate the total acidic groups. Five ml of sample solution were taken into a beaker and one ml of formalin solution (37%) was added. Then pH was brought to 7.0 with acid or alkali and the mixture was titrated with 0.01 mol sodium hydroxide solution. The end point of titration, pH 9.0, was measured by the glass electrode pH meter.

The amino groups were estimated by the ninhydrin colorimetric method of S. Moore and W. H. Stein[4] and its modified method of H. Rosen[5]. These results are shown in Tables I and II. Figures express moles per 10^4g of protein.

In addition to the quantitative determination described above, qualitative examination of the N-terminal amino acid of lysozyme was performed. The procedure was carried out as follows; an amount of twenty ml of irradiated solution containing 0.2 g of lysozyme was

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[1] Y. Obata and M. Koshika, *J. Agr. Chem. Soc. Japan*, **32**, 158 (1958).

[2] G. Alderton, *J. Biol. Chem.*, **164**, 1 (1946).

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[4] S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951); **176**, 367 (1948).

[5] H. Rosen, *Arch. Biochem. and Biophys.*, **67**, 10 (1957).

TABLE I. TOTAL ACIDIC GROUPS OF PROTEINS BEFORE AND AFTER THE IRRADIATION.
Moles per 10^4 g protein.

Proteins	No Irradiation	Irradiation
Lysozyme	4.80	5.00
α-Casein	17.00	16.90
β-Lactoglobulin	10.20	11.50

TABLE II. AMINO GROUPS OF PROTEINS BEFORE AND AFTER THE IRRADIATION.
Moles per 10^4g protein.

Proteins	No Irradiation	Irradiation
Lysozyme	2.00	2.00
α-Casein	5.50	5.60
β-Lactoglobulin	6.21	6.29
	5.75§	5.85§

§; measured by the method of H. Rosen.

evaporated to dryness in a vaccum desicator over phosphorous pentoxide. The residues were treated with 1-fluoro-2, 4-dinitrobenzene as usual way. DNP-Lysozyme was hydrolyzed in autoclave with 10 ml of 6 N hydrochloric acid at 105°C for ten hours. The hydrolysate was at first extracted with ethyl ether three times, and the water layer was concentrated and extracted with ethyl methyl ketone. Both extracts were tested by paperchromatography using *n*-butanol saturated with water as the solvent. Three spots in the ether fraction and one spot in the ethyl methyl ketone fraction were detected. These spots were compared with those of the authentic DNP-amino acids and identified as follows; dinitroaniline, di-DNP-lysine, dinitrophenol and ε-DNP-lysine. The R_F values of these spots are shown in Table III.

It has already been observed by A. R. Thompson⁶⁾ and W. A. Schroeder⁷⁾ that the N-terminal amino acid of lysozyme is lysine. Our results agree with them.

TABLE III. R_F VALUES OF AUTHENTIC DNP-AMINO ACIDS AND THOSE WHICH ARE DETECTED FROM THE HYDROLYZATE OF DNP-LYSOZYME

	R_F		
No Irradiation, Ether Ex.	0.63	0.73	0.905
〃 , Ethyl methyl ketone Ex.	0.32		
Irradiation, Ether Ex.	0.62	0.735	0.91
〃 , Ethyl methyl ketone Ex.	0.32		
di-DNP-lysine	0.73		
ε-DNP-lysine	0.32		
Dinitrophenol	0.63		
Dinitroaniline	0.90		

If the peptide bonds of protein are hydrolyzed by the ultraviolet light irradiation, there should be the formation of amino and carboxyl groups in addition to those originally present in the protein molecule. As shown in Tables I and II, however, there was no significant difference in the amount of both amino and carboxyl groups before and after the irradiation. Furthermore, in the qualitative determination of the N-terminal amino acid of lysozyme (Table III), there was detected no terminal amino acids than lysine originally present in native protein. S. Sakamura obtains similar results of α-casein and β-lactoglobulin. From these results, it is concluded that the protein peptide bonds are not hydrolyzed by the action of ultraviolet light and decomposition of tryptophan in the lysozyme as observed in our preceding investigation may occur without peptide bond cleavage.

With β-lactoglobulin, slight increase in acidic groups was observed after the irradiation. It is noted that the increase is probably ascribed to the transient phenolic groups rather than carboxyl groups formed by the hydrolysis of protein as indicated by J. S. McLean⁸⁾, because no concurrent increase in the amount of amino groups was observed.

Determination of Total Basic Groups. It has been observed that free amino acids are decomposed by the action of ultraviolet light and liberate ammonia. In connection with this, attempts to estimate the ammonia nitrogen and total basic groups including imidazole, guanidyl, α- and ε-amino groups were performed. The basic groups were determined by the method of H. Fraenkel-Conrat and H. Cooper⁹⁾ with the dye of orange G. In this case each protein was dissolved in citrate buffer (pH 2.2) and irradiated. Hydrolysis of protein was not observed in this pH region, also. One ml of solution containing 5 mg of protein was put into the several angle-head centrifuge tubes. To these tubes, increasing amounts (1, 2, 3, ····, ml) of 0.1 percent orange G solution were added. The suspensions were shaken mechanically for twenty hours. They were then centrifuged and aliquots of the supernatant solutions were diluted quantitatively. The color intensities at 480 mμ were determined by means of photoelectric colorimeter.

The dye concentration was calculated from the

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7) W. A. Schroeder, *J. Am. Chem. Soc.*, **74**, 281, 5118 (1952).

8) J. S. McLean, *J. Biol. Chem.*, **187**, 537 (1950).

9) H. Fraenkel-Conrat and H. Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

standard curve prepared with appropriate dilutions of orange G. The dye bound by the protein was determined by subtracting the excess found in the supernatant from the amount added. Results are shown in Table IV, in which figures express basic residues per 10^4g of protein.

Liberation of Ammonia. Ammonia nitrogen was determined by the diffusion method using the Conway's apparatus. The results are shown in Table V.

A little liberation of ammonia was observed and it is particularly noticeable that β-lactoglobulin liberates much more ammonia as compared with lysozyme and α-casein. It is probable from the model experiments, that the liberation of ammonia is due to the decomposition of amino acids such as tryptophan or histidine which has nitrogen atom not to participate in the peptide bonds of protein, although the difference in the amount of total basic groups in the proteins before and after the irradiation is little.

TABLE IV. TOTAL BASIC GROUPS OF PROTEINS BEFORE AND AFTER THE IRRADIATION. Basic residues per 10^4g protein.

Proteins	No Irradiation	Irradiation
Lysozyme	10.5	10.3
α-Casein	8.5	8.4
β-Lactoglobulin	10.1	10.4

TABLE V. LIBERATION OF AMMONIA FROM THE IRRADIATED PROTEINS. Results are expressed as moles per 10^4g protein.

Proteins	No Irradiation	Irradiation	NH_3 Liberated
Lysozyme	0.14	0.24	0.10
α-Casein	0.24	0.32	0.08
β-Lactoglobulin	0.25	0.63	0.38

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Studies on Koji

Part IV. Mold Protease Production by Replacement Culture and Inactive Form Protease in Cell Free Extract

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The effects of growth temperature on protease producing abilities of washed mycelial cells of *Aspergillus soyae* KS were investigated. The maximum ability of the cells grown at 35°C was almost the same as that of the cells grown at 20°C. Cells of 35°C culture rapidly lost their protease producing abilities after it reached its maximum, whilst cells of 20°C culture maintained their abilities for a comparatively long time until a later cultural time.

Effects of temperature on protease production of washed cells were the same as those obtained with growing cells. It was recognized that the protease activity of cell free extracts of mycelia increased during incubation. Thus it was supposed that there would be a zymogen precursor in the process of protease formation by this mold.

It has been reported that[1] the lower the cultural temperature of *Asp. soyae* KS, the larger is the amount of protease produced. In order to confirm this point, the increase of protease activity in washed cells from two growing cultures at 20°C and 35°C was compared. Sioda et al.[2] have shown that when washed cells of *Asp. oryzae* A-1-5, which scarcely produced protease in the growth medium, were shaken in water or phosphate buffer, a considerable amount of protease is produced. They suggested that[3] this condition would have forced mold cells to an abnormal biosynthesis[4,5] which competes with the normal protein synthesis, and protease would be produced by such an abnormal biosynthesis. Accordingly, it seemed possible to clarify the protease productivity of mycelia of growing cultures by comparing the increase in protease of washed cells during incubation under the same conditions with the initial culture. Subsequently, it is also found in this paper that when cell free extracts (C. F. E.) of *Asp. soyae* KS are incubated at various temperatures, the protease activity in the extracts are increased. This phenomenon was recognized only with the extracts of mycelia which were separated from the growing culture, actively producing much protease. From the results of experiments using C. F. E., it seemed that this mold must have a zymogen precursor as an intermediate in the process of protease formation. These results will be also reported here.

EXPERIMETAL

1. Strain used *Asp. soyae* KS was chosen as mentioned in the previous paper[1].

2. Culture Media Medium for paste culture was prepared by the same method as described in the previous report[6]. Four kinds of media were chosen for

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[1] K. Yamamoto, This Bulletin, **21**, 319 (1957).

[2] H. Sioda and K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **23**, 426 (1950).

[3] H. Sioda, *J. Agr. Chem. Soc. Japan*, **30**, 724 (1956).

[4] A. Matsushiro, T. Miwatani and D. Nakada, Biological Science (Tokyo) p. 7 (1956).

[5] M. Nomura and J. Hosoda, *J. Agr. Chem. Soc. Japan*, **30**, 233 (1956).

[6] K. Yamamoto, This Bulletin, **21**, 308 (1957).

TABLE I. COMPOSITION OF GROWTH MEDIA

component	A	B	C	D
	Kind of Medium (g/l)			
glucose	10	50	sucrose 30	sucrose 40
peptone	4.2	30	$NaNO_3$ 2	NH_4Cl 11
KH_2PO_4	30	5	K_2HPO_4 1	K_2HPO_4 0.4
$MgSO_4$	0.1	2.5	0.5	0.4
$ZnCl_2$, $FeSO_4$	trace	trace	trace	trace
NaOH	7			Na,K-tartrate 30

A, B and D medium are used in experiments of mold protease formation by K. Motonaga[7], H. Sioda et al.[2] and M.E. Maxwell[8], respectively, and C shows Czapek-Dox's medium.

liquid culture as shown in Table I[2,7,8], and a quantity of 150 cc of each liquid medium was taken into 500 cc flasks, and it was sterilized for 15 minutes under a steam pressure of 10 lb.

3. Replacement Culture The mycelial cells obtained from each culture were washed several times with distilled water, pressed with a filter paper, and placed in a double petri-dish not being brought into contact with any solution. The outside space of this double petri-dish was filled with distilled water in order to prevent the drying of washed mold cells. Then, the double petri-dishes containing the washed cells were kept at 35°C or 20°C.

4. Enzyme Preparation Appropriate amounts (20–60 mg) of the mycelial cells from paste culture, surface culture, or replacement culture, were mashed well in a glass-mortor with 20 cc of phosphate buffer (pH 7.0, M/15) and glass sand. After keeping the mashed cells for 4 hours at room temperature, the filtrate was taken and used as the enzyme preparation of each culture.

5. Determination of Protease Activity Protease activity of each preparation was measured in the same way as described in the previous report[1].

6. Cell Free Extracts and Determination of its Protease Activity The washed growing cells were pressed with a layer cloth to remove water and were mashed in a glass-mortor with the same volume of glass sand, and the homogenates were then extracted with two volumes of phosphate buffer (pH 7.0, M/15). The supernatant, being separated from the cell debris with freezing centrifugation (0°C, 10^5 r.p.m., 10 mins), was used as the cell free extracts.

Another preparation which was extracted from aceton-dried cells with 10 volumes of phosphate buffer was also used. All processes of C. F. E. preparation were carried out at 0°C. The C. F. E. was used immediately after preparation, and the protease activity in C. F. E. was determined at intervals by the usual method.

RESULTS

1. Increase of Protease Activity by Replacement Culture Method. Mycelial cells from paste cultures at 35°C and 20°C were washed well, and then these mycelia free from culture media were kept at 20°C and not brought in contact with any media. Fig. 1 shows that the protease activities in washed cells were increased, accompanying a definite increase in the spore number. It is also shown that any cell from cultures of various phase could attain its maximum protease activity within almost 48 hours' incubation after replacement.

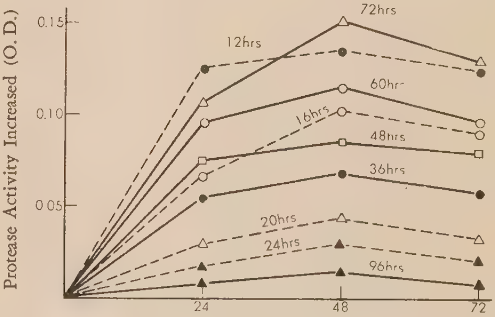

FIG. 1. Increase of Protease Activity in Washed Cell by Replacement Culture.

----- Cells from 35°C culture
——— Cells from 20°C culture

Figures (hrs) on the curves show a time of culture phase of washed cells.

Replacement culture was carried out under the condition which washed cells were kept at 20°C without touching any media: being common to Fig. 1, 2 and Table III.

2. Comparison of Protease Productivity between the two Mycelial Crops grown at 35°C and 20°C. The protease activity per mg of dried mycelial weight which increased during replacement culture (48 hrs, at 20°C) was used as a measures of protease productivity of the mycelium. As shown in Fig. 2, the protease producing ability of mycelium of 35°C culture taken at a certain phase of growth was equal

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7) K. Motonaga, read at the Meeting of J. Agr. Chem. Soc. Japan (1956).

8) M. E. Maxwell, *Aust. Sci. Res. Ser. B. Biol. Sci.*, **5** (1), 42 (1952).

to that of 20°C, that is, there was no significant difference between the maximum values of protease producing ability of mycelia from 35°C and those of mycelia from 20°C culture. However, a marked difference was observed in the protease producing ability of mycelium from the 35°C culture which decreased rapidly after its maximum, while the mycelium from the 20°C culture maintained such a high level of protease productivity until its later phase of growth. Moreover, the weight of mycelium from the 20°C culture was heavier than that from 35°C culture, when compared at the time producing the maximum level of protease (Fig. 2).

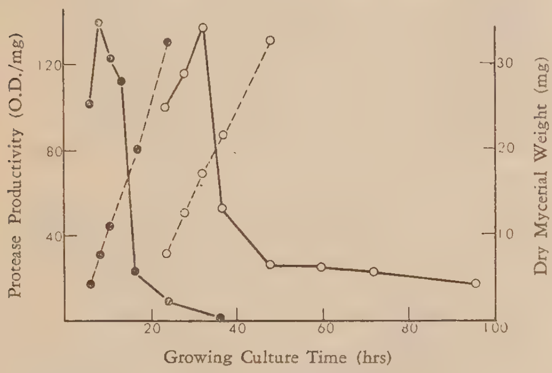

FIG. 2. Protease Productivities and Dry Weight of Washed Cells from 35°C and 20°C Culture.

—●— 35°C culture ——— Protease productivities
—○— 20°C culture - - - - - - Dry mycelial weight

The protease activity increased during replacement culture (48 hrs, at 20°C) per mg of dry mycelial weight was used as a measure of protease productivity of the washed cells.

3. Effect of the Temperature of Replacement Culture on Protease Production. The protease production of mycelia, which were harvested from the same growing culture and were secondary cultured in petri-dishes at different temperatures, was compared. Fig. 3 shows that protease production at 35°C was more rapid than that at 20°C, but after showing maximum activity the decrease in protease in the mycelium at 35°C was more rapid than that at 20°C. The maximum protease activity in the mycelia incubated at 35°C was lower than that at 20°C. These results with regard to the effect of the temperature of replacement culture were the same as those obtained with growing culture described in a previous report[1].

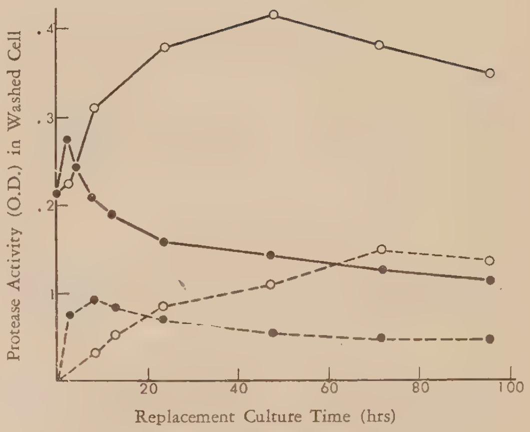

FIG. 3. Effect of Temperature of Replacement Culture on the Protease Production of Washed Cell.

—●— 35°C
—○— 20°C
- - - - - - - Cells from 35°C culture at 12 hrs. of growth phase
——— Cells from 20°C culture at 72 hrs. of growth phase

4. Increase of Protease Activity in Cell Free Extracts. The increase in protease activities of the cell free extracts by incubation was next investigated. The extracts were prepared from the mycelial cells which were grown on the four different media. From the results shown in Table II, it can be said that the protease activities of the extracts from mycelia which produced much protease in growth media were markedly increased during incubation, but the extracts from mycelia which produced little or no protease did not show such an increase. The protease productivities in replacement culture of the mycelia which have grown on mediums A and B are shown in Table III. It can easily be pointed out that the C.F.E. of mycelia grown on B medium at 24 hours of culture did not have the ability to increase protease during incubation, though

TABLE II. INCREASE OF PROTEASE ACTIVITY IN CELL FREE EXTRACTS FROM VARIOUS GROWING CELLS

kind of medium	cultural time (hrs)	pH of medium	mycelial weight (g)	protease in medium (O.D./g)	protease in mycelium	protease increased in C. F. E.
A	24	7.0	0.9	0.05	0.03	0.03
	48	7.0	2.0	0.090	0.02	0.03
	72	7.0	2.1	0.110	0.015	0.020
	96	7.0	2.0	0.110	0.010	0.015
B	24	5.0	0.8	0	0.010	0
	48	4.9	1.5	0	0.015	0
	72	4.8	3.0	0	0.020	0.040
	96	5.9	8.0	0.002	0.030	0.130
	120	7.6	9.0	0.010	0.060	0.080
C	48	6.6	1.5	0	0.005	0
	72	5.7	2.4	0	0.010	0
	96	6.5	3.0	0	0.010	0
D	24	5.4	1.0	0	0	0
	48	5.3	3.0	0.005	0.005	0.010
	72	5.6	4.0	0.005	0.020	0.030
	96	5.8	4.0	0.010	0.015	0.015

Values indicate optical density as protease activity: being common to Table II–VI.

TABLE III. INCREASE OF PROTEASE ACTIVITY OF WASHED CELLS FROM TWO CULTURES

medium	cultural time (hrs)	protease in medium	protease in mycelium	protease increased
A	24	0.050	0.067	0.134
	48	0.100	0.061	0.066
B	24	0	0	0.129
	48	0	0.021	0.030

the washed mycelia could produce a considerable amount of protease in replacement culture without added nutrients.

5. Some Characteristics of Increase in Protease Activity in C. F. E.

a) Kind of protease increased: It is now recognized that *Asp. soyae* KS produces three kinds of proteinase: alkaline, neutral and acid proteinase[9], and it is well known that the potato-inhibitor specifically inhibits the activity of alkaline protease[10,11]. By the addition of potato-inhibitor to C. F. E., it was proved that alkaline protease is the main component of protease which increased during incubation of C. F. E. (Table IV).

TABLE IV. EFFECT OF POTATO-INHIBITOR ON THE PROTEASE ACTIVITY INCREASED IN C. F. E.

	incubation time (mins)		
potato-inhibitor	0	90	180
none	0.030	0.072	0.156
added	0.015	0.013	0.010

Potato-inhibitor was prepared by Yasui's method. The pre-incubation was performed for 5 minutes at 30°C.

b) Effect of incubation temperature: The increase of protease activity in C. F. E. at various incubation temperatures was observed. It was found that C. F. E. could reach its maximum protease activity within 4 hours at 30°C and 48 hours at 0°C during incubation at pH 7.0 (Fig. IV).

c) Effects of inhibitors, ATP and amino acid mixture: The effects of the addition of inhibitors of protein synthesis, ATP or an amino acid mixture on the increase of protease activity in C. F. E. were then examined. These substances were found to have no effects on the increase of protease activity in C. F. E. (Table V).

d) Effects of C. F. E. on alkaline protease: It was investigated whether there was any

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9) K. Yamamoto, This Bulletin, **21**, 313 (1957).

10) K. Matsushima, *J. Agr. Chem. Soc. Japan*, **29**, 883 (1955).

11) T. Yasui, Lecture delivered at the meeting of J. Agr. Chem. Soc. Japan (1955).

change in the activity of alkaline protease when C. F. E. was added to alkaline protease solution.

The results showed that there was no difference between the protease activities with and without the addition of C. F. E. (Table VI).

e) Activities of C. F. E. with various concentrations of casein: The course of hydrolysis of casein by C. F. E. was examined (Fig. V) and it was found that C. F. E. hydrolysis casein of various concentrations show the same hydrolysis-curve.

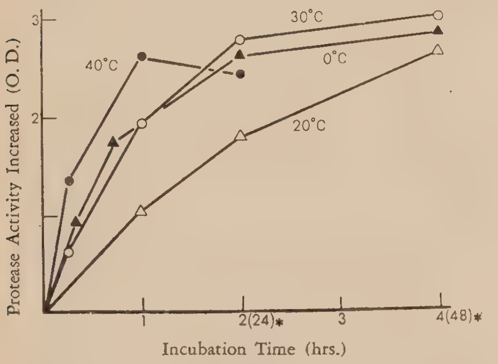

FIG. 4. Effect of Incubation Temperature on the Increase of Protease in C.F.E..

* Shows the incubation time (hrs.) for the incubation at 0°C.

Cell free extracts were incubated at various temperature and at pH 7.0.

TABLE V. EFFECT OF SOME SUBSTANCES ON THE INCREASE OF PROTEASE ACTIVITY IN C. F. E.

substances added		relative activity of increased protease (%)
none		100
2,4-DNP	final conc of 10^{-2} M	112
8-oxy-quinoline	//	82
chrolum phenycohl	//	103
NaN_3	//	96
Ethionine	//	103
8-aza-guanine	//	109
penicillin aureomycin streptomycin	//	103
amino acid mixture	//	101
A. T. P.	//	105

The cell free extract was incubated for 3 hours (pH 7.0, 30°C), after being mixted with each substance.

TABLE VI. EFFECT OF C. F. E. ON ALKALINE PROTEASE

composition of mixture (total volum of 10 cc) C.F.E.	alk. protease	protease activity measured	protease activity calculated	differentiation
5	5	0.226	0.230	−0.004
2.5	5	0.192	0.190	+0.002
5	0	0.070		
2.5	0	0.030		
0	5	0.160		

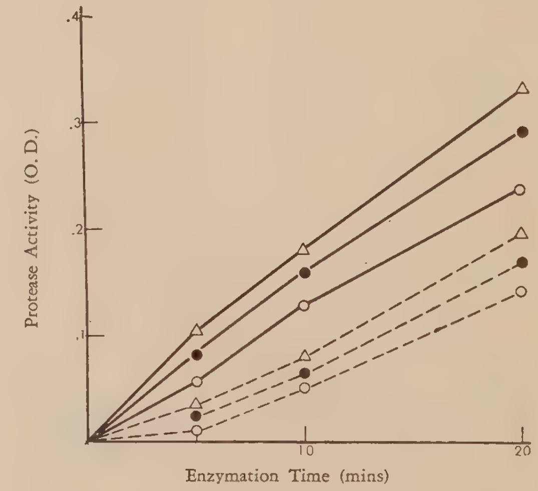

FIG. 5. Courses of Hydrolysis by C.F.E. and by Alkaline Protease on Various Concentration of Casein.

——— Alkaline protease ----- C. F. E.

△ 1% ● 2% ○ 4%

DISCUSSION

From the results obtained with washed mycelial cells, the presumption that the mycelium which had been cultured at lower temperature might have much stronger ability for protease production than that cultured at higher temperature was excluded. Both the mycelia from the 35°C culture and those from 20°C culture showed the same degree of ability to produce protease, when compared at certain phases of growth (Fig. 2). However, the total amount of protease produced was larger in the mycelia grown at lower temperatures.

Another difference between the 35°C culture and 20°C culture was that the protease productivity of the mycelia from 20°C lasted longer than that of the mycelia from 35°C. These facts might be attributed to the differences in the dry weight (mg) of mycelial cells: the dry weight of mycelia grown at 20°C is much larger than those grown at 35°C. In this paper, the increased protease activities during replacement culture were used as a measure of protease productivity. Then arises the question—what is the source of this increased protease in mycelial cells? Two possibilities are suggested: (1) the mycelia contain the precursor or (2) protease is newly synthesized by the cells upon incubation. The presence of zymogen precursors of protease is well known in animal bodies. Recently, zymogen precursors of Streptococcal proteinase and of Coccus P proteinase were also discovered by S.D. Elliott et al.[12] and L. Gorini et al.[13], respectively. Nomura et al.[14], and Fukumoto et al.[15] have also assumed the presence of some precursors in the process of amylase formation by *Bac. subtilis.* Working with *Asp. Kawachii,* Kanie[16,17] recognized the increase of amylase activity in C.F.E. and presumed the presence of a precursor of amylase in mycelial cells. However, no study has been made concerning the presence of the precursor of koji mold protease. From the results obtained in the present paper, it is concluded that there must be zymogen precursor in the process of mold protease formation.

That the protease is *de nove* synthesis of protein would not be probable, because the protease production was not inhibited by the addition of inhibitors of protein synthesis nor by the addition of metabolite analogues such as ethionone and 8-azoguanine (Table V). Since the mode of hydrolysis of casein by C.F.E. did not vary with increasing casein concentration (Fig. 5), it is not plausible that protease molecules in the C.F.E. are already conbined with some substrates which compete with casein. On the other hand, examination of the effects of incubation temperature on the mode of hydrolysis of casein by C.F.E. appeared to indicate that some kind of enzyme action is involved in the increase of protease activity of C.F.E. (Fig. 4). Thus, it seems that the protease activity increase in C.F.E. during incubation resulted from activation of the zymogen precursor which was contained in C.F.E.. As shown in Table II, all the C.F.E. prepared with mycelial cells which had been actively producing protease in growth media showed the same extent of increase in protease activity upon incubation. This would suggest that the zymogen precursor would not be stored in the mycelial cells during growth, but it is immediately transformed to the active enzyme as soon as it appears. On the other hand, the fact that the mycelial cells grown for 24 hours in medium B produced protease during replacement culture whereas incubation of C.F.E. of the same cells did not result in any increase in protease activity seems to show that the increased protease in the replacement culture was not attributable to zymogen precursor but was newly synthesized (Table II, III).

With regard to the zymogen precursor and its activating enzyme (kinase), further investigations are now in progress.

The author wishes to express his sincere thanks to Prof. K. Sakaguchi for his kind suggestions rendered throughout the course of this study, and to Dr. M. Nomura and Dr. F. Yoshida for their advice.

The author also wishes to thanks Dr. M. Mogi for kindly making many facilities available for the purpose of this study.

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12) S.D. Elliott and V.P. Dole, *J. Exp. Med.*, **85**, 305 (1948).

13) L. Gorini and G. Lanzavecchia, *Biochem. et Biophys. Acta*, **15**, 399 (1954).

14) M. Nomura, J. Hosoda, H. Yoshikawa and S. Nishimura, read at the International Symposium on E.C. (1957).

15) J. Fukumoto and T. Yamamoto, *Symposia on Enzyme Chemistry*, **7**, 3 (1952).

16) M. Kanie, *Memors of the Faculty of Agr. Kagoshima Univ.*, Vol. II p.35 (1956).

17) M. Kanie, Lectured at the meeting of J. Agr. Chem. Soc. Japan (1957).

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Comparative Studies of Brown Discoloration and Ammonia Liberation from the Milk Proteins by Ultraviolet Light Irradiation

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Investigations were undertaken on α-, β-casein, β-lactoglobulin and α-lactalbumin isolated from fresh milk to ascertain the development of color and ammonia liberation when exposed to ultraviolet light.

Experimental data showed that α-, β-casein equally contributed to cause considerable brown discoloration, followed by α-lactalbumin, while β-lactoglobulin remained almost colorless. Furthermore, additional evidence in a model system suggested that the participation of tryptophan is very important in such a discoloration.

On the other hand, some discussion is offered together with the suggestion that the liberation of ammonia might initiate from histidine residue without hydrolysis of linkage of peptide in protein.

In previous papers[1,2] attention has been drawn to the physico-chemical difference between the intact protein prepared from milk and denaturated protein exposed to UV-light.

In connection with the application of ionizing radiation for milk[3], the browning and the occurrence of objectionable aroma and flavors in the protein solution irradiated by UV-light has made it desirable to direct particular attention to this matter.

Thence, some investigators have noted on the color production in their proteins by irradiation, but the nature of the compounds responsible for such a color has not yet been elucidated. Similarly to the case of browning a few reports concerning the determination of ammonia formed free amino acids by UV-radiation have appeared but only few reports about proteins are available[4]. So it is of course, not proven what nitrogen compounds in protein are susceptible of the splitting off ammonia.

In this work, the following facts were confirmed. When exposed to UV-light, α- and β-casein are comparatively more responsible and α-lactalbumin is somewhat less, whereas β-lactoglobulin is almost not at all responsible for browning among the four representative proteins of cow's milk. With support from the result of model system experiments, the most important compound to which the browning may be ascribed is considered to be tryptophan residue rather than tyrosine or phenylalanine residue, but both of the latter can not be disregarded as a factor because they become susceptible to coloration only after tryptophan is eliminated.

It is also confirmed that lactose, which is the main carbohydrate in milk, does not cause any discoloration under the same conditions irrespective of the way it may be decomposed.

EXPERIMENTAL

Materials and Methods Fresh pooled whole-milk

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[1] Y. Obata and S. Sakamura, *J. Agr. Chem. Soc. Japan*, **32**, 251 (1958).

[2] S. Sakamura and Y. Obata, *J. Agr. Chem. Soc. Japan*, **32**, 667 (1958).

[3] For example, J. H. Werthein, R. N. Roychoudhury, J. Hoff, S. A. Goldblith, and B. E. Proter, *J. Agr. Food Chem.*, **5**, 944 (1957).

[4] A. D. Mclaren, *Advances in Enzymology*, **9**, 75 (1949).

taken from several cows was used throughout. α-, β-casein were prepared according to the method of Warner; their homogeneity was electrophoretically ascertained. β-Lactoglobulin was recrystalized two times and lyophilized for preservation until use. The purity of the protein has been described in the previous paper. α-Lactalbumin was purified the same run as used for the preparation of β-lactoglobulin according to the method of Aschaffenburg[5]. It was not crystallized but lyophilized following dialysis against distilled water in a celophan bag at room temperature for a long time and was made electrophoretically homogeneous under the applied condition (pH 6.8, phosphate buffer). In most of these experiments amino acids obtained from Ajinomoto Co. were used without further purification. All of the acetyl amino acid derivatives were prepared by using acetic anhydride and were checked by melting point. Glycyl-L-tyrosine and glycyl-L-tryptophan commercially available were purchased. Glutamine was isolated from beet roots according to the description of Vickery et al.[6] and identified by showing m. p. 184°. All other compounds were synthesized in this laboratory, particularly, L-isoleucyl-L-valine and its anhydride were synthesized by Mizutani[7].

Exposure to UV-light All of the samples were dissolved in phosphate buffer pH 6.8, ionic strength 0.1, to give a 1% concentration of protein or 0.2% of amino acid and amino acid derivatives individually. Each of the open Petri dishes (diameter, 50 mm) containing 10 ml of the solution was subjected to UV-radiation at a 150 mm distance under a light source of ACME HOME SUNLIGHT (100 v., 2 amp.) for fourteen hours. Temperature of the solution did not exceed above 15.

Measurement of Degree of Brown Color and Ammonia Liberation The samples radiated as described above showed a decrease in water content and so, before measurement distilled water was added to fill the contents up to the original level. Color measurement was carried out by comparing the absorption coefficient using a Beckman type spectrophotometer (Hitachi Co.) in the region of visible light.

Ammonia was colorimetrically determined by the micro-diffusion method with Conway's unit[8].

Qualitative Analysis of N-Terminal of Protein with DNFB* The dinitrophenyl derivatives of the proteins (α-casein and β-lactoglobulin) were prepared by Sanger's method[9]. Two hundred mg of protein dissolved in water or buffer, or 10 ml of irradiated solution was allowed to react with 0.5 ml of DNFB in ethanol containing 0.5 g sodium bicarbonate. The mixtures were shaken mechanically for four hours at room temperature. The resulting mixture was slightly adjusted to the acidic side by adding dilute hydrochloric acid. DNP-proteins precipitated were centrifuged, washed with ethanol and ether, finally dried in the exicator over phosphorous pentoxide in vacuo. DNP-protein was hydrolysed by autoclaving with 6 N hydrochloric acid at 110° in a sealed glass tube for ten hours. The hydrolysate was divided into the ether-soluble fraction and the residue (water-soluble fraction). The latter was evaporated to dryness under reduced pressure and redissolved with water or extracted with ethyl methyl ketone. Both fractions were individually chromatographied using filter paper[10] and silica gel which was made of sodium silicate[9]. Authentic DNP-amino acids employed for comparison were also synthesized and purified by the present authors.

RESULTS AND DISCUSSION

Brown Discoloration. It must be stated that lactose did not cause any coloration in these model experiments. As shown in Fig. 1, all of the proteins contributed to the yellowish brown discoloration by exposure to UV-light. But the order of color intensity was as follows: α-, β-casein > α-lactalbumin > β-lactoglobulin; the last one scarcely became brown. Thus, it was presumed that such a discoloration might be attributable to amino acids. The effect of UV-light exposure on various amino acids and on their derivatives was therefore compared under the same conditions. The results are shown in Fig. 2. Tryptophan, even the amino group of which some members were acetylated, was most intensively brown-colored. When compared with glycyltyrosine and glycyltryptophan, the latter became definitely much more

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[5] R. Aschaffenburg and J. Drewry, *Biochem. J.*, **65**, 273 (1957).

[6] H. B. Vickery, G. W. Pucher and H. E. Clark, *J. Biol. Chem.*, **109**, 39 (1935).

[7] Y. Obata and J. Mizutani, in press.

[8] B. E. J. Conway, Micro-diffusion Analysis and Volumetric Error, 2nd. ed., London, Crosby, Lockwood and Sons.

* The following abbreviations in this paper indicate, DNP: 2, 4-dinitrophenyl group; DNFB: 1, 2, 4-dinitrofluorobenzene.

[9] F. Sanger, *Biochem. J.*, **39**, 507 (1949). S. Mizushima and S. Akabori, Chem. of Protein, Kyoritsu Shuppan Co., Vol. 4 (1956) p. 209.

[10] E. F. Melon, A. H. Korn and S. R. Hooper, *J. Amer. Chem. Soc.*, **75**, 1675 (1953).

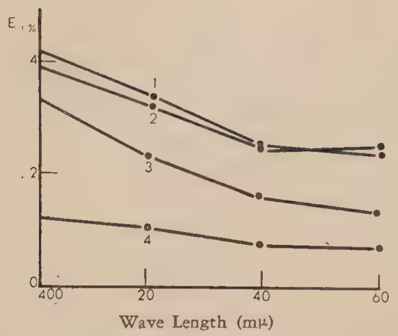

FIG. 1. Comparison of the Color Development from the Proteins in 1% Solution in Phosphate Buffer (pH 6.8) upon UV-Radiation.

1) α-Casein	3) α-Lactalbumin
2) β-Casein	4) β-Lactoglobulin

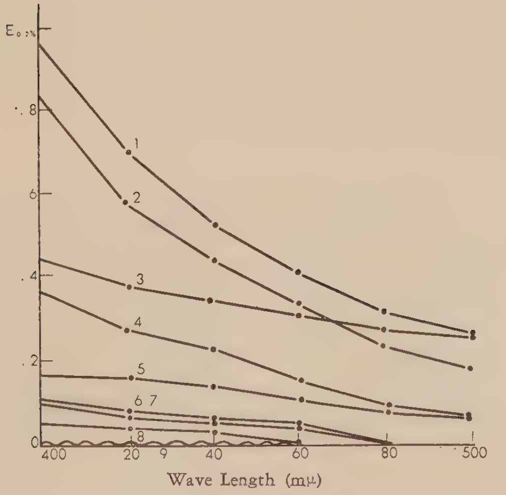

FIG. 2. Comparison of the Color Development from the Various Amino Acids and their Derivatives in 0.2% in Phosphate Buffer (pH 6.8) upon UV-Radiation.

1) Tryptophan	6) Cystine
2) N^{α}-Acetyltryptophan	7) Histidine
3) Tyrosine	8) Hydroxyproline
4) Phenylalanine	9) Other Amino Acids
5) N-Acetyltyrosine	

brown than the former.

These results suggest that tryptophan residue is a most important factor for the browning, though tyrosine and phenylalanine may participate partly in the discoloration only after tryptophan is eliminated. However, there is a fact that β-lactoglobulin hardly causes such a discoloration in spite of the existence of tryptophan. This point, now seems to be explicable as a protective action in sulfhydryl group of peptide chain or as protective influence due to some unknown spatial arrangement.

Liberation of Ammonia. As shown in the previous paper, the present authors observed that the liberàtion of ammonia from β-lactoglobulin undoutedly took place, even though ammonia was obtained from α-casein in the experiment which was carried out in detail

TABLE I. YIELDS OF AMMONIA OBTAINED FROM AMINO ACIDS AND OTHER RELATED COMPOUNDS IN 0.2% IN PHOSPHATE BUFFER (pH 6.8) UPON UV-RADIATION

Compounds	NH_3(μMol/ml)
Glycine	0.50
Alanine	0.70
Threonine	0.64
Valine	0.59
Leucine	0.46
*Cystine	0.94
Methionine	0.68
Proline	0.37
Glutamine	0.48
Asparagine	0.45
Glutamic Acid	0.37
Aspartic Acid	0.36
Butyramide	0.13
Valeramide	0.25
Phenylalanine	0.30
*Tyrosine	0.40
Tryptophan	0.20
Histidine・HCl	1.40
Lysine・HCl	0.64
Arginine	0.33
Acetylglycine	0.15
N-Acetyltyrosine	0.33
N^{α}-Acetyltryptophan	0.15
Isoleucylvaline	0.45
*Isoleucylvaline Anhydride	0.19
**β-Lactoglobulin	0.30
**α-Casein	0.19
Skim Milk	1.13

*. . .used as suspension
**. . .used in 1% solution

later. Thus, in order to establish what kind of nitrogen-containing compound in protein is responsible for the liberation of ammonia, determinations of free ammonia were made on solutions of various amino acids and their derivatives after exposure as described above. The results of such determinations are shown in Table I, data of which are presented with an increased amount of ammonia liberated upon irradiation. Histidine produced the greatest amount of ammonia, followed by cystine, methionine etc., whereas amino acid amide liberated was in an extremely small extent. On the other hand, ammonia from β-lactoglobulin was calculated to be ca 1 mol per molecule on the basis of 35,000 molecular weight[11]. Therefore, it seems to be possible that liberation of ammonia in the protein mostly originates from histidine and only partly from others.

DNP-Amino Acid If the peptide linkage of protein is hydrolysed on UV-radiation as was postulated by Carpenter[12], the hydrolysation is most likely expected to result in alternation on the N-terminal amino acids of the protein in both qualitative and quantitative sence. As shown in Tables II and III, DNP-amino acid was identified as DNP-leucine and ε-DNP-lysine in both intact and irradiated β-lactoglobulin, and as ε-DNP-lysine only in both cases of casein, although their values were not determined.

TABLE II. RESULTS OF PAPER CHROMATOGRAPHY OF DNP-AMINO ACIDS FOUND IN α-CASEIN IRRADIATED AND NOT IRRADIATED

	R_F			
Sample	Solvent A		Solvent B	
Irradiated, ether soluble	0.85	0.64	0.95	0.25
〃 , water soluble	0.57		0.07	
Not irradiated, ether soluble	0.85	0.64	0.95	0.25
〃 , water soluble	0.57		0.08	
Dinitroaniline 1*	0.85		0.95	
Dinitrophenol 2*	0.64		0.25	
Di-DNP-lysine 3*	0.78		0.72	
DNP-arginine 4*	0.63		0.135	
ε-DNP-lysine 5*	0.57		0.07	

solvent A; *n*-butanol saturated by distilled water
solvent B; the upper solution of *n*-butanol, *n*-butylacetate, 1% ammoniumhydroxide (1;2;3)

1*…m. p., 176–8, 2*…m. p., 112–3, bleached by exposure of the paper to HCl vapor, 3*…m. p., 173, 4*…m. p., 234. 5 *…m. p., 187, $C_{12}H_{16}O_6N_4 \cdot HCl \cdot H_2O$, ninhydrin reaction positive.

TABLE III. RESULTS OF PAPER CHROMATOGRAPHY OF DNP-AMINO ACIDS FOUND IN β-LACTOGLOBULIN IRRADIATED AND NOT IRRADIATED

	R_F	
Sample	Solvent A	Solvent B
Irradiated, ether soluble		0.94, 0.51, 0.31
〃 , water soluble	0.67	0.064
Not Irradiated, ether soluble		0.94, 0.51, 0.31
〃 , water soluble	0.66	0.06
Dinitroaniline		0.94
Dinitrophenol		0.30
DNP-Leucine*		0.51
ε-DNP-lysine	0.67	0.059

*…m. p., 93
Also see the explanatory notes of Table II.

Further research efforts were directed toward paper chromatographic identification of the radiation-breakdown products from model compound, such as glycyltyrosine, glycyltryptophan, in order to obtain additional evidence whether hydrolysis of peptide linkage occurs

TABLE IV. RESULTS OF PAPER CHROMATOGRAPHY OF COMPOUNDS INDUCED FROM GLYCYL-L-TYROSINE AND GLYCYL-L-TRYPTOPHAN BY EXPOSURE TO UV-LIGHT

	R_F		
	Solvent (1)		Solvent (2)
Sample irradiated	Ninhydrin	Rolin R.	Ninhydrin
Glycyltyrosine	0.44, 0.29	0.44, 0.31	0.11, 0.04
Glycyltryptophan	—	—	—
Authentic Sample for comparison Tyrosine	0.43	0.43	0.27
3, 4-Dihydroxy phenylalanine	0.26	0.28	0.06
Tryptophan	0.50	0.50	0.62
Glycine	0.19		
Glycyltyrosine	0.44	0.44	0.11
Glycyltryptophan	0.55	—	0.46

solvent (1)…*n*-butanol, acetic acid, water, (4:1:5)
solvent (2)…*m*-cresol saturated by water

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11) B. L. Larson and R. Jenness, *Biochem. Prep.*, **4**, 23 (1955).
12) D. C. Carpenter, *J. Amer. Chem. Soc.*, **62**, 289 (1940).

or not. The results are shown in Table IV. The radiation conditions were essentially the same but on a smaller scale than that described in the section of the present paper, that is, 2 mg of sample was contained in 10 ml distilled water, exposure for four hours, followed by concentration at reduced presure at room temperature. No spot of expected amino acid from hydrolysis of glycyltyrosine was detected, but an unidentified spot different from that of the original peptide, not found in the control, was detected on the paper chromatograms. It gave a blue color with Folin reagent[13], and a positive reaction with ninhydrin, the color of which was somewhat yellow and after only a little while turned purple resembling that of the original peptide.

Accordingly, the above facts suggested the occurrence of an oxidized compound maintaining the peptide linkage such as glycyl-3,4-dihydroxyphenylalanine. On the other hand, in the case of glycyltryptophan neither a newly produced spot nor the original spot could be detected on paper chromatograms by either reaction but the browning of the solution was more intense than that resulting from glycyltyrosine.

Concerning the effect of UV-radiation on free tyrosine and tryptophan, the above described observations led to the detection of dopa and kynurenine individually, in accordance with the case of exposure to sunlight[13,14].

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[13] Y. Obata and S. Sakamura, *J. Agr. Chem. Soc. Japan*, **27**, 769 (1953).

[14] S. Sakamura and Y. Obata, *J. Agr. Chem. Soc. Japan*, **29**, 616 (1955).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2, p. 121~125, 1959]

Isolation and Identification of L-Isoleucyl-L-valine Anhydride from the Soy-bean Meal Hydrolysates (AMINOSAN-EKI)

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L-Isoleucyl-L-valine anhydride was synthesized from L-isoleucine and L-valine. The comparison between the diketopiperazine derivative which had been isolated from soy-bean meal hydrolysates and the authentic sample of L-isoleucyl-L-valine anhydride showed that the both compounds were identical.

INTRODUCTION

In the previous paper[1], it was reported that a diketopiperazine derivative, isolated from the soy-bean meal hydrolysates (AMINOSAN-EKI) was proved as L-isoleucyl-L-valine anhydride by elemental analysis, melting point, and by paper chromatography. In the present paper L-isoleucyl-L-valine anhydride was synthesized and the isolated diketopiperazine derivative was compared with synthesized L-isoleucyl-L-valine anhydride.

L-Valyl-L-isoleucine was synthesized by the use of phthalyl-L-valine[2]. This method has been conveniently used because the intermediates are mostly crystalline, and the optically active products are obtained.

At first the method of K. Yamashita and M. Yashiro[3] was applied, but the preparation of phthalyl-L-valyl chloride was unsuccessful.

J.C. Sheehan and G.P. Hess[4] have described a new and very useful method of forming peptide bonds, that is, the two components, one containing a free carboxyl group and the other a free amino group, are coupled directly and rapidly in high yield by treatment with N,N′-dicyclohexylcarbodiimide at room temperature.

This method was successfully applied, and phthalyl-L-valyl-L-isoleucine ethyl ester was obtained in high yield (98%) from phthalyl-L-valine and ethyl L-isoleucinate on treatment with N,N′-dicyclohexylcarbodiimide in tetrahydrofuran for four hours at room temperature.

L-Isoleucyl-L-valine anhydride was prepared by the usual method from L-valyl-L-isoleucine.

EXPERIMENTAL AND RESULTS

1. L-Isoleucine. L-Isoleucine was separated from the leucine fraction by the method of Y. Tsuchiya and H. Tsukagoshi[5]. The leucine fraction, raw material, was generously supplied by Ajinomoto Co., Tokyo.

At first, the concentration of the isoleucine fraction (over 80%) was carried out in several steps, and then 4-nitro-4′-methyl-diphenylamine-2-sulfonate of L-isoleucine, obtained by the reaction with 4-nitro-4′-methyl-diphenylamine-2-sulfonic acid, was recrystallized and separated from L-leucine sulfonate (L-isoleucine 4-nitro-4′-methyl-diphenylamine-2-sulfonate, m.p. 189–190°).

Sodium 4-nitro-4′-methyl-diphenylamine-2-sulfonate was prepared by the method of F. Ullmann and K. Jüngel[6], and F. Ullmann and R. Dahmen[7]. The yield of 2-chloro-5-nitrobenzene sulfonic acid, m. p. 167–170° (decomp.); reported[8], m. p. 165–170° (decomp.), was 56% and of sodium 4-nitro-4′-methyldiphenylamine-2-sulfonate was 92%.

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[1] Y. Obata and J. Mizutani, This Bulletin; **22**, 14 (1958).

[2] S. Akabori and S. Mizushima, Chemistry of protein (Kyoritsu Publishing Co. Ltd.), (1) p. 658 (1954).

[3] K. Yamashita and M. Yashiro, *J. Agr. Chem. Soc. Japan*, **28**, 674 (1954).

[4] J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

[5] S. Akabori and S. Mizushima, Chemistry of protein (Kyoritsu Publishing Co. Ltd.), (1) p. 74 (1954); Y. Tsuchiya and H. Tsukagoshi, Japanese Patent 16,407 (1952); Y. Tsuchiya, Japanese Patent 2,325; 2,326 (1952).

[6] F. Ullmann and K. Jüngel, *Ber.*, **42**, 1077 (1909).

[7] F. Ullmann and R. Dahmen, *Ber.*, **41**, 3750 (1908).

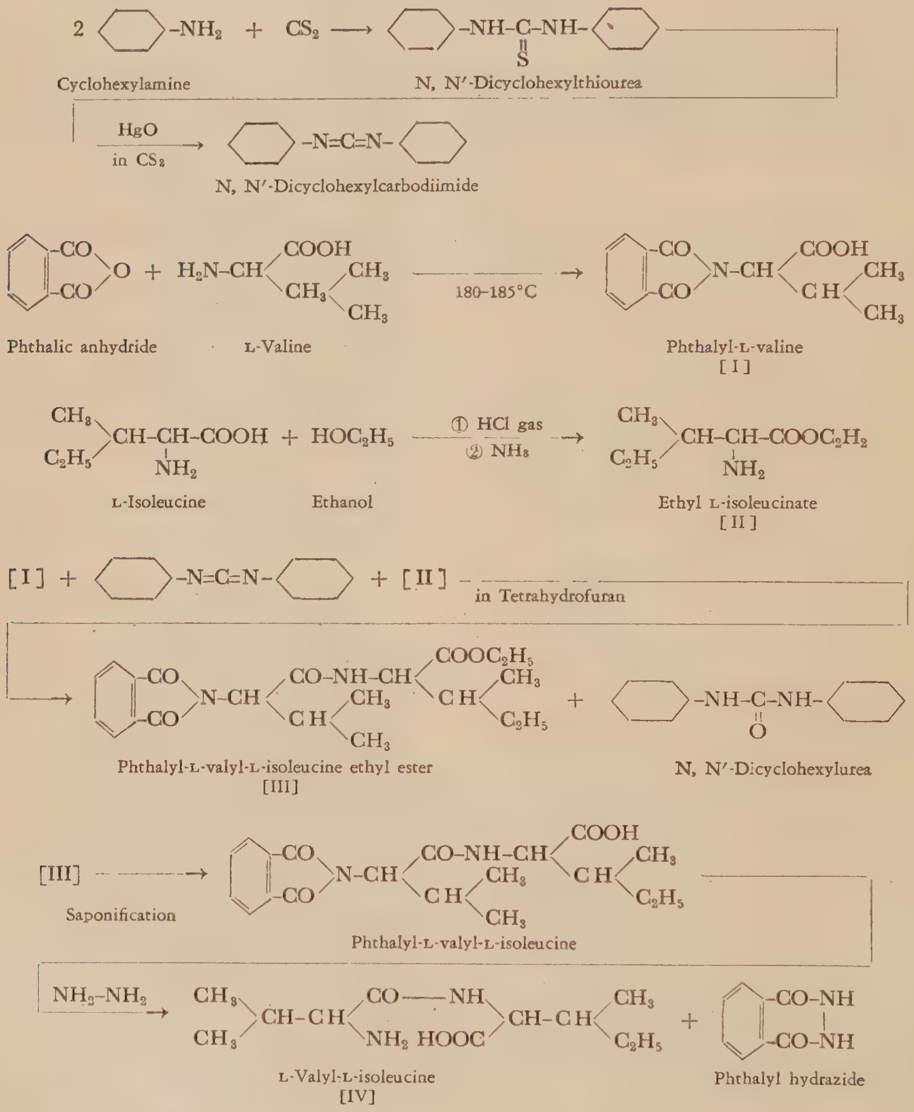
Leucine and isoleucine hydrochlorides (805 g) were obtained from 644 g of the leucine fraction, and 36.0 g of crude isoleucine was obtained from 684 g of leucine and isoleucine hydrochlorides.

Crude isoleucine (30 g) gave 6.7 g of pure L-isoleucine; sublimes at 168–170°, m. p. 284° (decomp.), $[\alpha]_D^{25}=+41.2°$ (20% hydrochloric acid), reported[5], $[\alpha]_D^{20}=+40.7°$ (20% hydrochloric acid).

R_F value of L-isoleucine separated is shown in Table I.

2. L-Valine. Authentic L-valine was kindly supplied by Ajinomoto Co., Tokyo.

3. Synthesis of L-Isoleucyl-L-valine Anhydride.



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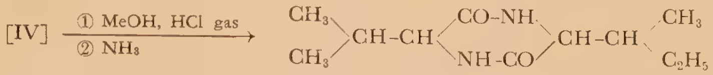
8) *Beilsteins Handbuch*, **11**, 73 (1928).



L-Isoleucyl-L-valine anhydride

FIG. 1. Outlines of the Synthesis of L-Isoleucyl-L-valine Anhydride.

Preparation of N, N′-dicyclohexylthiourea[9]. Eight grams of N, N′-dicyclohexylthiourea (65.6%) were obtained from 10 g of cyclohexylamine (b. p. 132–3°; reported[10], 134.5°) and 10 g of carbon disulfide, and m. p. 180–181°.

Preparation of N, N′-Dicyclohexylcarbodiimide. N, N′-Dicyclohexylcarbodiimide was prepared by the method of R. Herbeck and M. Pezzati[11]. The yield was 50.7%; b. p. 154–156°/11 mm.

Preparation of Phthalyl-L-valine. Phthalyl-L-valine was synthesized from L-valine and phthalic anhydride by the method of I. Schumann and R. A. Boissonnas[12]. The yield was 67%; m. p. 115–116°.

Preparation of Ethyl L-Isoleucinate. Two grams of L-isoleucine were suspended in 100 ml of absolute ethanol and dry hydrogen chloride gas was bubbled in for saturation with ice cooling. The solvent was evaporated in vacuo at room temperature, and then esterification was repeated five times. At last, the solvent was removed completely and the residue was suspended in 20 ml of chloroform, and to this was added dropwise the chloroform solution containing 2.5% of dry ammonia with ice cooling. After ten minutes, deposits of ammonium chloride were filtered off, and the solvent was evaporated completely under reduced pressure at room temperature. The yield of ethyl L-isoleucinate was 1.9 g (80%).

Preparation of Phthalyl-L-valyl-L-isoleucine Ethyl Ester. Phthalyl-L-valine (2.95 g), 3 g of N, N′-dicyclohexylcarbodiimide and 1.9 g of ethyl L-isoleucinate were dissolved in 50 ml of tetrahydrofuran (b. p. 65.4°), and reacted for four hours at room temperature. The mixture was shaken occasionally, and after four hours 3 ml of glacial acetic acid were added to decompose the excess reagent. The insoluble N, N′-dicyclohexylurea was removed, the solvent was evaporated under reduced pressure, the residue was dissolved in 50 ml of ethyl acetate, and insoluble matters were filtered off. The ethyl acetate solution was washed with 1 N sulfuric acid and 5% aqueous solution of potassium bicarbonate, and dried with anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the crude crystals were obtained in 98% yield (4.5 g) on the addition of petroleum ether.

Preparation of Phthalyl-L-valyl-L-isoleucine. The crude ester (4.5 g) mentioned above was treated with the mixture of 36 ml of dioxane and 24 ml of 1N sodium hydroxide for four hours at room temperature. After then, the solvents were evaporated under reduced pressure below 35°C, and the residue was dissolved in water. After filtration the solution was acidified with 24 ml of 1 N hydrochloric acid. The separated oily substance was extracted with ethyl acetate, the ethyl acetate solution was washed with water, dried with anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was dissolved in a small amount of ethyl ether and recrystallized by the addition of petroleum ether; yield 4 g (95%), m. p. 85–7° (uncorr.).

Preparation of L-Valyl-L-isoleucine. The mixture of phthalyl-L-valyl-isoleucine (4 g), 99% ethanol (60 ml) and 1.4 g of hydrazine-hydrate (80%) was refluxed on the boiling water bath for an hour. And then, the solvent was evaporated to dryness under reduced pressure, 50 ml of water were added, brought to pH 5–6 with dilute acetic acid, and heated for one hour on the boiling water bath. Water was then evaporated to dryness under reduced pressure, the residue was dissolved in 30 ml of butanol, and after cooling the insoluble phthalyl hydrazide was removed. The solvent was evaporated to dryness under reduced pressure. In order to remove phthalyl hydrazide completely, column chromatography using cellulose powder was carried out. The column with 4.5 cm in diameter and 30 cm in height was prepared, and butanol-acetic acid-water (4 : 1 : 2) was used as the solvent.

The fraction containing L-valyl-L-isoleucine was collected, and the solvents were evaporated completely under reduced pressure. The residue was dissolved in ethanol, the ethanol solution was decolorized with active carbon, evaporated, and recrystallized by the addition of ethyl acetate; the yield of L-valyl-L-isoleucine 1.5 g (58%), m. p. 308–310° (uncorr.).

The R_F value of L-valyl-L-isoleucine is shown in

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[9] A. Skita and H. Rolfes, *Ber.*, **53**, 1247 (1920).

[10] The Merck Index (Merck & Co., Inc.), p. 299 (1952).

[11] R. Herbeck and M. Pezzati, *Ber.*, **71**, 1938 (1938).

[12] I. Schumann and R. A. Boissonnas, *Helv. Chim. Acta*, **35**, 2237 (1952).

Table I.

TABLE I. THE R_F VALUES OF L-VALYL-L-ISOLEUCINE AND RELATED COMPOUNDS BY PAPER CHROMATOGRAPHY

	R_F
L-Valyl-L-isoleucine	0.89
Leucyl-valine	0.85
L-Leucine	0.68
L-Isoleucine	0.67
L-Valine	0.55

Solvents: BuOH-AcOH-H_2O (4:1:2).

Preparation of L-Isoleucyl-L-valine Anhydride. One gram of L-valyl-L-isoleucine was suspended in 30 ml of absolute methanol, dry hydrogen chloride was passed in for saturation with ice cooling. The solvent was then evaporated under reduced pressure below 25°C, and the esterification was repeated twice. After then, the evaporation under reduced pressure was repeated many times to remove hydrogen chloride as completely as possible. The residue was dissolved in a sufficient amount of dry methanol, the solution was poured little by little with ice cooling into 30 ml of dry methanol solution saturated with ammonia at zero degree.

After keeping one week at 27°C, crude needles deposited. The product was recrystallized from 50% acetic acid, and fine needles, as shown in Fig. 2, were obtained; yield 0.5 g (55.6%), m.p. 308–310°, $[\alpha]_{\mathrm{D}}^{19}=-42°$ (1% glacial acetic acid solution).

Anal. Found: C, 62.79; H, 9.45; N, 12.89%.
Calcd. for $C_{11}H_{20}O_2N_2$: C, 62.23; H, 9.50; N, 13.20%.

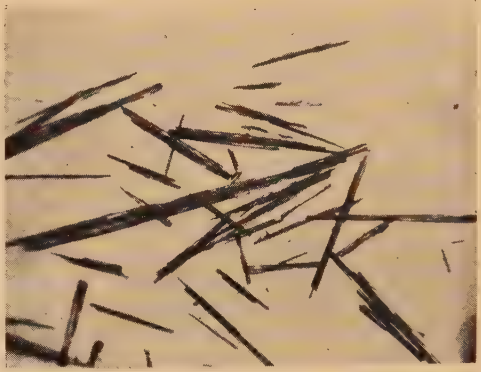

FIG. 2. Synthesized L-Isoleucyl-L-valine Anhydride

Comparison between isolated diketopiperazine derivative and synthesized L-isoleucyl-L-valine anhydride is shown in Table II and Fig. 3.

TABLE II. COMPARISON BETWEEN THE PROPERTIES OF ISOLATED DIKETOPIPERAZINE DERIVATIVE, SYNTHESIZED L-ISOLEUCYL-L-VALINE ANHYDRIDE, *d*-ISOLEUCYL-*d*-VALINE ANHYDRIDE REPORTED BY DAKIN, AND *l*-LEUCYL-*d*-VALINE ANHYDRIDE REPORTED BY ABDERHALDEN AND FUNK, AND BY FISCHER AND SCHEIBLER

	m.p.	$[\alpha]_D$	Mixed m.p.
Synthesized L-Isoleucyl-L-valine Anhydride *Anal.* C H N Calcd.: 62.23 9.50 13.20% Found: 62.79 9.45 12.89%	308–310°	−42° (19°C, AcOH)	Showed no depression
Isolated Diketopiperazine Derivative Found: 62.41 9.64 12.77%	308–310°	−42° (19°C, AcOH)	
d-Isoleucyl-*d*-valine Anhydride Isolated by H. D. Dakin[13]	310–312°	−43.5°, −42.0° (20°C, AcOH)	
l-Leucyl-*d*-valine Anhydride Isolated by Abderhalden, Funk[14]	280–285°	−44.27° (20°C, AcOH)	
Synthesized by Fischer, Scheibler[15]	282°	−50.2° (25°C, AcOH)	

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[13] H. D. Dakin, *Biochem. J.*, **12**, 314 (1918).
[14] E. Abderhalden and C. Funk, *Z. physiol. Chem.*, **53**, 19 (1907).
[15] E. Fischer and H. Scheibler, *Ann.*, **363**, 136 (1908).

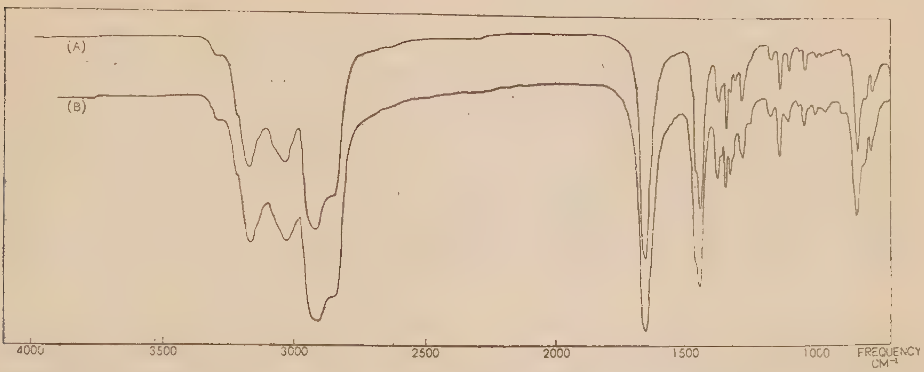

FIG. 3. Comparison of the Infra-red Spectra of Isolated Diketopiperazine Derivative with Those of Synthesized L-Isoleucyl-L-valine Anhydride in Nujol Mull.

(A) Synthesized L-Isoleucyl-L-valine Anhydride.
(B) Isolated Diketopiperazine Derivative from AMINOSAN-EKI.

Acknowledgements The authors wish to express their thanks to Ajinomoto Co., Tokyo, for kindly supplying us the precious samples. The authors' thanks are also extended to Mr. M. Izawa of the Central Research Institute, Japan Monopoly Corporation, Tokyo, for the infra-red spectro-analysis, and to the Department of Agricultural Chemistry, University of Tokyo, for the microanalysis.

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[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2, p. 126~130, 1959]

Pseudomonas Pigments. III.

Derivatives of Pyoluteorin*

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Received August 4, 1958

Esters and methyl derivatives of a new pigment pyoluteorin, produced by a strain of *Pseudomonas aeruginosa* have been synthesized, and catalytic hydrogenation of the derivatives was carried out to give the corresponding dechloro compounds. It has become clear that pyoluteorin is composed of an unsaturated nucleus and has two phenolic hydroxyls, one amino and the other a cross-conjugated carbonyl group.

In the previous communication[1)] it was shown that two strains of *Pseudomonas aeruginosa*, T 359*** and IFO 3455, when grown on bouillon medium containing calcium chloride, produced a new pigment pyoluteorin $C_{11}H_7O_3NCl_2$, which has strong inhibitory action on the growth of a number of bacteria and protoza. Since these findings were considered to be the first recorded instance of bacteria giving a chlorine containing metabolic product, it was deemed essential that pyoluteorin should be identified structually.

As it was shown in the earlier communication[1)], pyoluteorin (I) has no C-CH_3, N-CH_3, or OCH_3 group and gives a pKa of 8.1 in 50% ethanolic solution by titration and a pKa of −1 and 12 by the spectroscopic method, indicating the presence of two acidic groups and a weak basic group. The pKa values suggested that the acidic function might be phenolic. This was also presumed from the strong absorption at 3310 cm^{-1} in the infrared spectrum (Fig. 1) and the bathochromic shift in the ultraviolet absorption spectra (Fig. 2).

In order to identify the acidic groups, pyoluteorin (I) was acetylated with acetic anhydride in benzene to form an O, O′-diacetate (II), $C_{11}H_5ONCl_2(OCOCH_3)_2$. II still has a strong absorption band at 3240 cm^{-1} in the infrared spectrum which is supposed to be due to the NH group. Both pyoluteorin and its O, O′-diacetate, on benzoylation with benzoyl chloride in pyridine, yielded a tribenzoate (III), $C_{11}H_4ONCl_2(COC_6H_5)(OCOC_6H_5)_2$, and a diacetylmonobenzoate (IV), $C_{11}H_4ONCl_2(COC_6H_5)(OCOCH_3)_2$, showing no absorption band of OH and NH groups in the infrared spectrum respectively. Further, when II or III was hydrolyzed with potassium hydrogen carbonate, I was recovered.

When I was methylated with diazomethane in ether or with dimethyl sulfate in alkaline solution, a trimethyl compound (V), $C_{11}H_4ONCl_2(CH_3)(OCH_3)_2$, was obtained which also had no absorption band due to OH and NH groups in the infrared spectrum. As a result of analysis, V was found to have two OCH_3 groups and one N-CH_3 group. II, on methylation with diazomethane in dry ether, gave an O, O′-diacetyl-N-methyl compound (VI), $C_{11}H_4ONCl_2(CH_3)(OCOCH_3)_2$, showing no absorp-

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* A preliminary report of this investigation is published in *J. Am. Chem. Soc.*, **80**, 4749 (1958).

** Juso, Nishino-cho, Higashiyodogawa-ku, Osaka, Japan.

1) R. Takeda, *J. Ferm. Tech.*, Osaka, **36**, 281 (1958).

*** Later, it was also shown that phenazine-1-carboxylic acid, oxychlororaphine[2)], anthranilic acid[3)] and 2-heptyl-3, 4-dihydroxyquinoline[4)] were produced in the cultures of the strain.

2) R. Takeda, *ibid.*, **36**, 286 (1958).

3) R. Takeda and I. Nakanishi, *ibid.*, **37**, 54 (1959).

4) R. Takeda and I. Nakanishi, *ibid.*, **37**, 59 (1959).

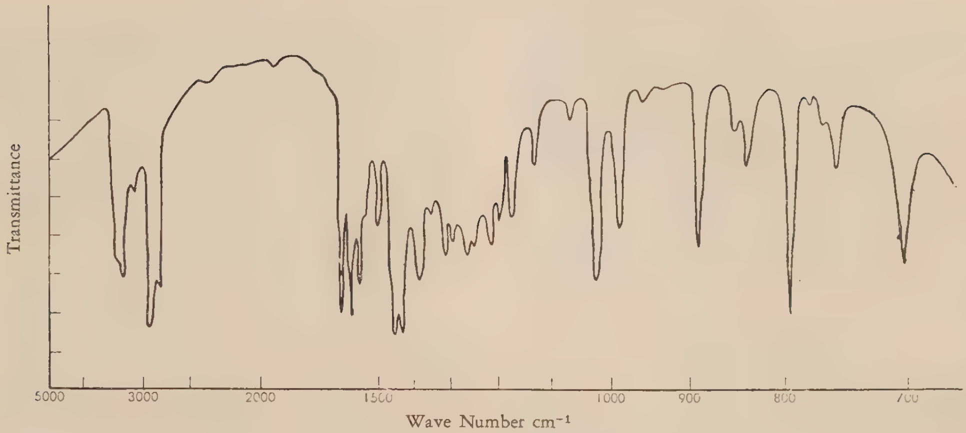

FIG. 1. Infrared Spectrum of Pyoluteorin

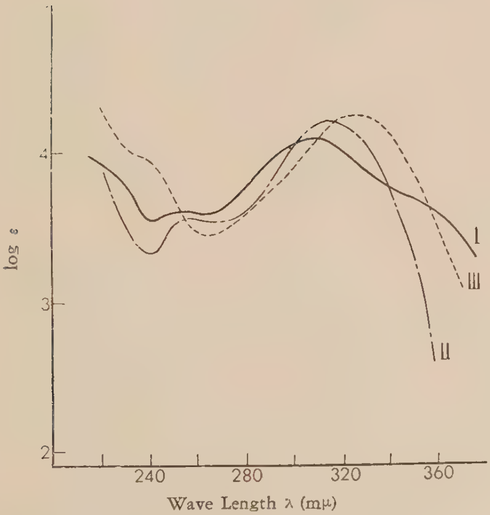

FIG. 2. Ultraviolet Spectra of Pyoluteorin

I in Ethanol
II in 0.01 N HCl
III in 0.01 N NaOH

-tion band around 3000 cm^{-1} in the infrared spectrum. On the other hand, when II was methylated with diazomethane in alcoholic solution containing water, methyl groups giving V were substituted for the two acetyl groups. Replacement of an acetyl group of certain phenolic acetoxy compounds with a methyl group by the action of diazomethane was reported⁵⁾ and the present author also confirmed this reaction by converting phenyl acetate to anisole applying the same conditions as in the case of methylation of II. Pyoluteorin (I), II and V on hydrogenation over palladium-charcoal in alcoholic solution containing magnesium oxide⁶⁾ or triethylamine as chlorine-acceptor yielded dechloropyoluteorin (VII), dechloro-O, O′-diacetylpyoluteorin (VIII) and dechlorotrimethylpyoluteorin (IX), respectively. Upon hydrolysis with potassium hydrogen carbonate, VIII also gave VII. On the other hand, when II was hydrogenated with palladium-charcoal catalyst in acetic acid containing a theoretical amount of sodium acetate as the chlorine-acceptor, the product, after hydrolysis with potassium hydrogen carbonate, was identified as a monodechloropyoluteorin (X). Unexpectedly, VII, on methylation with diazomethane in dry ether, gave an O, O′-dimethyl compound (XI), $C_{11}H_7ON(OCH_3)_2$, which had a strong absorption band at 3230 cm^{-1} in the infrared spectrum indicating the presence of NH group. The different behavior found between I and VII towards methylation with diazomethane strongly suggests that the chlorine atoms increase the acidic nature of the NH group. All of the derivatives obtained above exhibited an absorption around 1640 cm^{-1} indicating the presence of a cross-conjugated

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5) J. Herzig and Tichatschek; *Ber.*, **39**, 268 (1906).

6) N. Whittaker; *J. Chem. Soc.*, **1951**, 1565.

C=O group in their infrared spectra and an absorption maxima around 300 mμ in their ultraviolet spectra. It has therefore become evident, that pyoluteorin is composed of an unsaturated nucleus having two phenolic OH groups, one NH and one cross-conjugated C=O group as its functional groups.

Further work on the chemical structure of pyoluteorin is now in progress and the results will be reported later.

EXPERIMENTAL*

Acylation.

a) O, O′-Diacetylpyoluteorin (II).

i) With Acetic Anhydride in Benzene: I (0.2 g) was refluxed for three hours with acetic anhydride (0.4 ml) in anhydrous benzene (1.6 ml). After removal of the solvent and excess acetic anhydride, the product was crystallized from acetone as colorless needles, yield 0.25 g. When recrystallized from acetone/petroleum ether, a portion of 0.2 g of the material of *analytical quality* was obtained. m. p. 207–208°. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 250 mμ (ε 4,630), 313 mμ (ε 16,100). ν (NH) 3240, ν (ester C=O) 1760, 1735, γ (cross-conj. C=O) 1640 cm^{-1}. *Anal.* Found: C, 50.53; H, 3.07; N, 3.83; Cl, 20.00; $2\,COCH_3$, 23.81. Calcd. for $C_{15}H_{11}O_5NCl_2$: C, 50.58; H, 3.11; N, 3.93; Cl, 19.91; $2\,COCH_3$, 24.17.

ii) With Acetyl Chloride in Pyridine: I (0.27 g) was dissolved in pyridine (5 g) and acetyl chloride (0.35 g) was added. After standing for two days at room temperature the reaction mixture was dissolved in ether. The ether solution was washed successively with 5% hydrochloric acid solution, 1% sodium hydrogen carbonate solution and water and was dried over anhydrous sodium sulfate. After removal of the ether, the residue was crystallized from acetone/petroleum ether, yield 0.2 g. m. p. 205–206°. The product was identified as the diacetate by comparison of the infrared spectra and determination of mixed melting point.

b) Tribenzoylpyoluteorin (III).

Pyoluteorin (I) (0.2 g) was dissolved in pyridine (5 g) and benzoyl chloride (0.4 g) was added, and the mixture was allowed to stand for three days at room temperature. The dark brown reaction mixture was dissolved in ether. After washing with 5% hydrochloric acid, 1% sodium hydrogen carbonate solution, and with water and subsequently drying over anhydrous sodium sulfate, the solution was evaporated *in vacuo* and the residue was crystallized from ether/petroleum ether as a pale yellow crystal, yield 200 mg. On recrystallization from ether 100 mg of the material was recovered. m. p. 158°. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 238 mμ (ε 41,700), 315 mμ (ε 11,000). ν (ester C=O) 1730, ν (cross-conj. C=O) 1640 cm^{-1}, no OH band, no NH band. *Anal.* Found: C, 65.77; H, 3.37; N, 2.25; Cl, 11.99. Calcd. for $C_{32}H_{19}O_6NCl_2$: C, 65.76; H, 3.28; N, 2.40; Cl, 12.13.

c) O, O′-Diacetylmonobenzoylpyoluteorin (IV).

II (0.2 g) was dissolved in pyridine (5 g) and benzoyl chloride (0.2 g) was added and the mixture was kept standing overnight at room temperature. Fifty milliliters of water was then added to the resulting solution and the solution was extracted with three portions of ether (50 ml). The ether solution was washed with 5% hydrochloric acid, 1% sodium hydrogen carbonate solution, and water and concentrated to dryness *in vacuo* and then the product was recrystallized from methanol, yield 200 mg. m. p. 140°. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 255 mμ (ε 18,500), 310 mμ (ε 11,300); ν (ester C=O) 1765, 1735, ν (cross-conj. C=O) 1640 cm^{-1}, no OH band, no NH band. *Anal.* Found: C, 57.68; H, 3.47; N, 2.84; Cl, 15.69. Calcd. for $C_{22}H_{15}O_6NCl_2$: C, 57.41; H, 3.29; N, 3.04; Cl, 15.41.

Hydrolysis of the Esters.

a) Pyoluteorin (I) from its O, O′-Diacetate (II).

II (0.2 g) was dissolved in methanol (10 ml) and 20% potassium hydrogen carbonate solution (5 ml) was added and the mixture heated on a water-bath for one hour. After removal of the solvent, the remaining solution which was turbid with a yellow precipitate was extracted with ether and the ether solution was washed with water and dried over anhydrous sodium sulfate. The ether was removed *in vacuo* and the residue was crystallized from acetone/petroleum ether as sulfur yellow fine needles, m. p. 174° (dec.), yield 110 mg. The product was identified as pyoluteorin by comparison of their infrared spectra.

b) Pyoluteorin (I) from Tribenzoylpyoluteorin (III).

The tribenzoate (III) (0.2 g) was hydrolyzed in the same way as mentioned above to yield original pyoluteorin, m. p. 182° (dec.). yield 50 mg.

Methylation.

a) Trimethylpyoluteorin (V).

i) Methylation of Pyoluteorin with Dimethylsulfate in Alkaline Solution: I (0.5 mg) was dissolved in acetone (5 ml) and dimethyl sulfate (5 ml) was added. Then, eight lots of 5 ml of 2 N sodium hydroxide solution were added at intervals with mechanical stirring.

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* All melting points are uncorrected, and the infrared spectra were measured in Nujol mulls.

After standing for several hours at room temperature, the pale yellow product which separated out from the alkaline solution, was filtered, washed and dried, yield 0.55 g. Two recrystallizations from methanol yielded trimethylpyoluteorin as colorless prisms, m. p. 135–136°, yield, 0.4 g. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 260 mμ (ε 5,900), 305 mμ (ε 17,000). ν (cross-conj. C=O) 1640 cm^{-1}, no OH band, no NH band. *Anal.* Found: C, 53.28; H, 4.15; N, 4.63; Cl, 22.51; 2 OCH$_3$ 19.49; NCH$_3$, 4.44. Calcd. for $C_{14}H_{13}O_3NCl_2$: C, 53.52; H, 4.17; N, 4.46; Cl, 22.57; 2 OCH$_3$, 19.75; NCH$_3$, 4.78.

ii) Action of Diozomethane on Pyoluteorin in Dry Ether: I (0.5 g), on methylation with an excess of diazomethane in dry ether gave the same methylated product as that in the methylation with dimethyl sulfate in alkaline solution, m. p. 135–136°, yield 0.3 g.

iii) Action of Diazomethane on II in Methanol Containing Water: II (0.5 g) was dissolved in methanol (40 ml) containing water (8 ml). On treatment with an excess of diazomethane, constant and steady evolution of gas was observed. The reaction mixture was allowed to stand for four days. After removal of the ether from the reaction mixture the residue was crystallized from methanol, yield 200 mg. m. p. 135–136°. *Anal.* Found: C, 53.34; H, 4.28; N, 4.58. Calcd. for C, 53.52; H, 4.17; N, 4.46. The product was proved to be identical with V.

iv) Action of Diazomethane on Phenyl Acetate; Formation of Anisole: To an ethereal solution of diazomethane (400 ml) was added phenyl acetate (10 g) dissolved in methanol (40 ml) containing water (8 ml). The solution was allowed to stand at room temperature for two days and concentrated *in vaeuo*. Distillation of the residue gave 5.0 g of a colorless liquid, b. p.$_{40}$ 75–77°, together with 3.8 g of the starting material. The infrared spectrum of the former was identical with that of anisole. The reaction did not occur when it was conducted in the absence of water.

b) O, O′-Diacetyl-N-methylpyoluteorin (VI).

II (0.9 g) was dissolved in dry ether (60 ml) and the solution was treated with an excess of diazomethane in ether for two hours at room temperature. After removal of the ether, a solid separated out, yield 0.5 g. It crystallized as colorless needles, m. p. 157–158°, from acetone/petroleum ether. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 255 mμ (ε 5,340), 310 mμ (ε 16,800). ν (ester C=O) 1753, ν (cross-conj. C=O) 1638, ν (ester C–O) 1178 cm^{-1}, no OH, no NH band. *Anal.* Found: C, 51.95; H, 3.34; N, 3.67; Cl, 19.30; 2 COCH$_3$, 22.98; NCH$_3$, 3.81. Calcd. for $C_{16}H_{13}O_5NCl_2$: C, 51.91; H, 3.54; N, 3.78; Cl, 19.16; 2 COCH$_3$, 23.26; NCH$_3$, 4.06.

c) O, O′-Dimethyldechloropyoluteorin (XI).

VII (0.1 g) was methylated with an excess of diazomethane in dry ether. After standing for 24 hours, the reaction mixture was evaporated *in vacuo*. The product was crystallized from methanol, yield 50 mg. m. p. 187–188°. ν (NH) 3230, ν (cross-conj. C=O) 1615 cm^{-1}. *Anal.* Found: C, 67.38; H, 5.45; N, 6.33; 2 OCH$_3$, 26.55. Calcd. for $C_{13}H_{13}O_3N$: C, 67.52; H, 5.67; N, 6.06; 2 OCH$_3$, 26.84.

Catalytic Hydrogenation.

a) Dechloropyoluteorin (VII).

(I) (0.5 g), dissolved in absolute ethanol (60 ml) and triethylamine (2 ml), was shaken in an atmosphere of hydrogen with palladium-charcoal prepared from palladium chloride (0.3 g) and charcoal (2.0 g). The uptake of hydrogen was very rapid, being almost completed within one hour and a half and the volume of the gas absorbed corresponded to two times that of the theoretical. After separation of the catalyst, the alcoholic solution was concentrated *in vacuo*. To the residue were added ether and water, and the ether layer was washed successively with 5% hydrochloric acid, 1% sodium hydrogen carbonate solution and water and dried over anhydrous sodium sulfate. After removal of the solvent, the product was crystallized from ether/petroleum ether as colorles needles, m. p. 141–142°, yield 120 mg. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 298 mμ (ε 15,400). ν (OH, NH) 3400, 3250, ν (cross-conj. C=O) 1615 cm^{-1}. *Anal.* Found: C, 64.83; H, 4.54; N, 6.72. Calcd. for $C_{11}H_9O_3N$: C, 65.02; H, 4.46; N, 6.89. The dechlorination seemed to be attended with further degradation judged by the hydrogen and the yield. This was also observed in the case of b).

b) Dechloro-O, O′-diacetylpyoluteorin (VIII).

II (1.4 g), dissolved in absolute ethanol (200 ml) and triethylamine (4 ml), was hydrogenated over palladium-charcoal. The uptake of hydrogen was almost completed within one hour and a half and the volume of the gas absorbed corresponded to one and a half times of the theoretical. The catalyst was filtered off and the alcoholic solution was evaporated *in vacuo*. To the residue were added ether and water, and after the ether layer was washed in the same manner as described above the product was crystallized from ether/petroleum ether as needles, m.p. 110°, yield 400mg. $\lambda^{\mathrm{EtOH}}_{\mathrm{max.}}$ 302 mμ (ε 15,600), ν (NH) 3270, ν (ester C=O) 1755, ν (cross-conj. C=O) 1615 cm^{-1}. *Anal.* Found: C, 62.55; H, 4.63; N, 4.90. Calcd. for $C_{15}C_{13}O_5N$: C, 62.71; H, 4.56; N, 4.88.

VIII (0.5 g) was dissolved in methanol, and potassium hydrogen carbonate (2.5 g) and water (10 ml) were added and the mixture was refluxed for one hour. After addition of water (20 ml), the solvent was removed *in vacuo* and the product was extracted with ether. The ether solution was washed with water and dried over anhydrous sodium sulfate. The product was crystallized from ether/petroleum ether as colorless needles, m. p. 142–143°, yield 100 mg. *Anal.* Found: C, 64.75; H, 4.29; N, 6.67. Calcd. for $C_{11}H_9O_3N$: C, 65.02; H, 4.46; N, 6.89. The infrared spectrum of the product was superimposable on that of VII.

c) Monodechloropyoluteorin (X).

II (0.7 g), dissolved in glacial acetic acid (80 ml) containing sodium acetate (0.93 g), was hydrogenated over palladium-charcoal. The product was crystallized in colorless needles, m. p. 156–158°. The product (0.2 g) was refluxed with potassium hydrogen carbonate (1.0 g) in methanol containing water for one hour. After treatment of the reaction mixture in the usual manner monodechloropyoluteorin was crystallized from ether as yellow needles, m. p. 196–197°. $\lambda_{max.}^{EtOH}$ 300 mμ (ε 13,000). ν (OH, NH) 3290, ν (cross-conj. C=O) 1630 cm^{-1}. *Anal.* Found: C, 55.53; H, 3.28; N, 5.73; Cl, 14.57. Calcd. for $C_{11}H_8O_3NCl$: C, 55.59; H, 3.39; N, 5.89; Cl, 14.92.

d) Dechlorotrimethylpyoluteorin (IX).

V (0.25 g) was dissolved in ethanol (80 ml) containing magnesium oxide (0.32 g) and the solution was shaken in the atmosphere of hydrogen with palladium-charcoal for 3 hours. The hydrogen uptake was almost completed within one hour and a half. The catalyst, magnesium chloride and excess magnesium oxide were filtered off, and after removal of the solvent, the residual product was crystallized from methanol in colorless prisms, m. p. 137°, yield 150 mg. $\lambda_{max.}^{EtOH}$ 295 mμ (ε 18,200). ν (cross-conj. C=O) 1630 cm^{-1}, no OH band, no NH band. *Anal.* Found: C, 68.68; H, 6.10; N, 5.98; 2 OCH_3, 25.01; NCH_3 5.82. Calcd. for $C_{14}H_{15}O_3N$: C, 68.55; H, 6.16; N, 5.71; 2 OCH_3, 25.30; NCH_3, 6.13. On hydrogenation with Raney nickel and triethylamine, trimethylpyoluteorin gave the same product as in the case of hydrogenation with palladium-charcoal and magnesium oxide, their identity being verified by comparison of their infrared absoption spectrum and by mixed melting point determination.

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Studies of Macerating Enzyme acting on Middle Lamella Pectin

Part IV. Action of Macerating Enzyme on Pectic Substances

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Cl. felsineum var. *sikokianum* produces macerating enzyme and polygalacturonase. Macerating enzyme was separated from the cultural solution of the bacterium by using the ion-exchange resin, Duolite CS-101. When macerating enzyme acted on the barks of Ganpi, this enzyme hydrolyzed pectin in the barks, and the ratio of decrement of pectin was found to be 22 per cent of pectin contained in the plant materials. It was also found that macerating enzyme attacked pectic substances prepared from various plant materials. The enzyme hydrolyzed pectic acid more slowly than pectin, and was found to be inactive on methylglycoside of polygalacturonic methyl ester.

Already, a great deal of knowledge concerning polygalacturonase (PG) and pectinesterase (PE) is available since the properties of these enzymes and the conditions of their activities have been reported, and McColloch and Kertesz have reported on their removal of PE from commercial pectinases by the use of ion-exchange resins[1].

However, knowledge in regard of the pectic enzymes other than PG and PE is very scanty. Seegmiller and Jansen[2] reported the existence of polymethylgalacturonase (PMG) which hydrolyzed the glycosidic linkages of the esterified galacturonic acid residues, and Roboz et al.[3] studied on pectin depolymerase (PDP) which caused diminution of viscosity of pectic acid and pectin, and hydrolysis of uronide linkages.

Kertesz suggested that the existence of a definite enzyme protopectinase is doubtful[4].

The author has reported in the previous papers[5,6] that macerating enzyme (ME) and PG could be separated by the use of ion-exchange resin, and in the present paper, experiments on ME acting on middle lamella pectin and on free pectin are reported. As a result, it is concluded that ME is classified as a pectinase, but is different from PG which reveals easily hydrolysis of pectic acid, and it is also different from PMG or PDP.

EXPERIMENT AND RESULTS

The cultivation of the bacterium was performed by the method mentioned in the previous paper[7].

The activity of PG was determined by measurement of the decrease in viscosity caused by the enzyme action at 37°C, for 1 hr. observed in the reaction media containing 0.5 per cent pectic acid. The activity was expressed as the "Abbauzahl" A, described by Weber, Deuel[8] and Roboz et al.[3].

The activity of ME was represented as the degree of separation of fibres from plant materials caused by the action of the enzyme on plant pectin. The activity of ME on free pectin was expressed in terms of the increase in reducing value, decrease of pectin and by change in viscosity. The reaction media for these experiments contained 0.5 per cent pectin, and the reducing value was determined by a modification of the Willstätter, Schudel hypoiodite method, the amount of pectin subsequently determined with the method of Nanji and Norman9).

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[1] R. J. McColloch and Z. I. Kertesz, *J. Biol. Chem.*, **160**, 149 (1945).

[2] C. G. Seegmiller and E. F. Jansen, *ibid.*, **195**, 327 (1952).

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The pH value of all the reaction media was adjusted to 6.0 by the addition of phosphate buffer, and reaction was performed at 37°C.

I. Separation of Macerating Enzyme and Polygalacturonase.

(1) Separation of macerating enzyme: Cultural solution of *Cl. felsineum* var. *sikokianum* was centrifuged, and the resulted clear broth was passed through a column of Duolite A-7 for the purpose of removing colouring matter and some part of impurities. The passed solution, the unadsorbed part of the broth, was employed in the present experiments as original enzyme solution.

A column of 20 ml of Duolite CS-101 was buffered with phosphate buffer solution, pH 7.2. Two hundred ml of the original enzyme solution was passed down through the resin column, and the enzymes were adsorbed on the ion-exchange resin. After the adsorption was completed, the column was washed with water, and then N/10 hydrochloric acid solution was added to the column in order to elute ME. One fraction of the passed solution was adjusted to 20 ml, and thus elution of ME was performed. The activities of PG and ME in each of the twenty fractions were determined. Fractions which contained only ME were collected and dialyzed against distilled water at 5°C, for 48 hrs. This procedure has been described in detail in the previous paper6).

It was found that the separated ME has the following properties. (a) It was completely inactivated when the enzyme solution was kept at 70°C, for 20 min. or at 100°C, for 5 min. (b) The optimum pH of this enzymatic action was 6.0. (c) Biuret, Xanthoproteic, and Cole reactions were all proved to be positive. (d) Its action was inhibited by Ag and Pb ions. (e) When ME was migrated by using a paper-electrophoretic method, it ran towards the cathode. As shown in Fig. 1., ME appeared as a single band when it was stained by bromphenol blue. The portions which corresponded to the band were cut, and ME was eluted with water from these paper strips. This solution acted on barks of Ganpi, and the separation of fibres from the barks was observed after 24 hours' reaction. (f) The author has reported that *Cl. felsineum* var. *sikokianum* did not produce PE, in the previous paper10) relating to the studies on the retting of plant fibre materials for Japanese paper manufacture. In order to confirm the nonexistence of PE in ME solution, activity of PE was observed with the procedure of a modified Kertesz' method11). The solution of pectic enzymes which was obtained by extraction from commercial pectinases; a preparation of crude enzymes produced by Penicillia, was used for comparative experiments. Ten ml of ME solution or 5 ml of the mold enzyme soln. was added to 15 ml of 1 per cent citrus pectin or Mitsumata pectin solution, and reaction mixtures were kept at 37°C, for 20 hrs. A distinct increase in acidity was observed on the reaction medium of mold enzyme, while no increase was detected in the solution in which ME and pectin existed. From these results it was found that this ME preparation have no action on PE. (h) ME did not act on xylan and soluble starch, and had no action on cells of *Micrococcus lysodeikticus*.

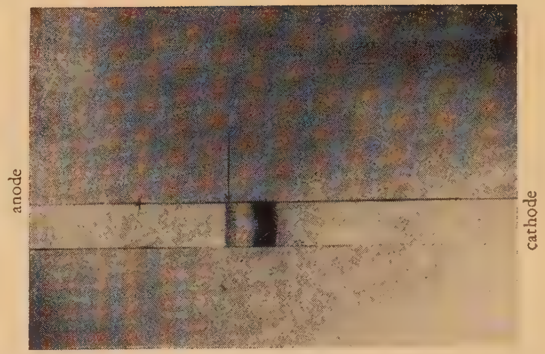

FIG. 1. Photograph of Paper Electrophoresis of Macerating Enzyme.

The arrow head indicates apex. Buffer was mixed soln. of M/50 $NaH_2PO_4 \cdot 2H_2O$ and M/50 $Na_2HPO_4 \cdot 2H_2O$, its pH value was adjusted to 7.4. Electrophoresis was conducted at potential gradients of 24 V/cm., giving a current of 0.15 mA/cm., and at 25°C, for 4 hrs.

(2) Maceration action of macerating enzyme on several plant materials: Action of ME was observed by the use of barks of Ganpi, Kōzo and Mitsumata, tubers of potato and sweet potato, roots of carrot and radish, and a segment of Natsudaidai orange. Barks were cut in size of 1 × 1 cm, other plant materials in 1 × 1 × 0.1 cm, and one piece of each material was subjected for the test of maceration action. Amounts of 0.25, 0.5 or 1.0 ml of the ME solution was added to the substrate of plant materials respectively. The

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TABLE I. ACTION OF MACERATING ENZYME ON VARIOUS PLANT MATERIALS

Plant material	Degree of maceration action			
	ml (0)	ml (0.25)	ml (0.5)	ml (1.0)
Barks of Ganpi	–	++	++++	+++++
Barks of Mitsumata	–	+	++	+++
Barks of Kōzo	–	+	++	+++
Potato	–	+++++	+++++	+++++
Sweet potato	–	+	++	+++
Carrot	–	–	+	+++++
Radish	–	+	+++	+++++
Segment of Natsudaidai	–	–	+	++

enzyme reactions were continued for 20 hrs., and then the materials were washed with water, and the degree of maceration observed. The results are shown in Table I, the number in brackets representing volume (ml) of the enzyme solution employed in the reaction media.

As shown in Table I, ME of the bacterium could macerate the barks of plant fibre materials, tubers of potatoes and roots of vegetables, and also represent the action on a segment of orange.

(3) Separation of polygalacturonase: PG was separated from the mixed enzyme solution according to the procedure mentioned in the previous paper5). PG solution reduced the viscosity of pectic acid solution, and its activity was found to be 25.5 per cent A, when PG acted on 0.5 per cent pectic acid solution for 1 hr.

II. Action of Macerating Enzyme on the Barks of Ganpi.

When ME acted on the barks of Ganpi, decomposition of middle-lamella pectin was observed. Ganpi, *Wikstroemia sikokiana* Fr. et Sav., is a raw material of Japanese paper, and white barks of this material were employed as a sample in the present experiment. The barks were cut in size of 1×1 cm, one piece weighing about 0.05 g, and 0.5 g of barks were added to ME solution, and macerating treatment was continued at 37°C. The reaction media contained 10 ml of ME solution 10 ml of distilled water, 5 ml of McIlvaine's phosphate buffer solution (pH 6.0), and 0.25 ml of toluol. The barks were taken out from reaction media at the reaction periods of 4, 8 and 16 hrs., pressed slightly, and washed with a definite volume of water. The washed water was added to reaction media, and the amount of pectin in the resulted reaction media was determined. Fibres obtained from the barks were dried, cut into small pieces, and the amount of pectin in the fibres was determined. The results are shown in Table II.

The barks of Ganpi, 0.5 g, were taken into a flask containing 25 ml of M/30 ammonium oxalate solution, and the flask kept at 85°C for 1/4 to 3 hrs., then 0.5 g of the same barks was added to 25 ml of EDTA (ethylenediamine tetraacetate) solution, and the reaction continued at 37°C for 4 to 20 hrs. In these reactions the separation of fibres was observed, and dissolved, and residual pectin were assayed. The results are shown in Table II.

TABLE II. MACERATION ACTIONS OF MACERATING ENZYME, AMMONIUM OXALATE AND OF EDTA ON BARKS OF GANPI

Reagents	Time of reaction hrs.	Separation of fibres	Amount of pectin after reaction			
			in reaction media and washed soln. (A) mg	in separated fibres (B) mg	recovered pectin (A+B) mg	yield of recovered pectin %
Macerating enzyme	4	+++	5.4	32.7	38.1	91.1
	8	++++	6.9	26.8	33.7	80.6
	16	+++++	12.5	20.1	32.6	78.0
M/5 EDTA	4	++	21.2	19.8	41.0	98.1
	8	+++	20.1	24.8	44.9	107.4
	20	++++	20.1	21.9	42.0	100.5
M/30 NH_4-oxalate	0.25	++	17.8	24.9	42.7	102.2
	1	++++	22.5	19.7	42.2	101.0
	3	+++++	26.5	18.7	45.2	108.0
Blank test	16	–	0	41.8	41.8	

By the action of ammonium oxalate and EDTA, pectin which was insoluble in water was converted into a soluble form, and the loss of pectin was not found through the process. On the other hand, 78.0 to 91.1 per cent of middle lamella pectin was recovered in the reaction media in which ME attacked the barks of Ganpi. Therefore, middle lamella pectin was not converted into the water soluble form, but hydrolyzed by the enzyme.

III. Action of Macerating Enzyme of Pectin Obtained from Various Plant Materials.

Ganpi pectin was prepared according to the following procedure. Pectin was extracted from the barks of Ganpi with ammonium oxalate solution, then the extracted pectin solution was dialyzed, and pectin was precipitated by the addition of ethanol. The gel of pectin was dissolved in water, the ethanol precipitation procedure was applied again, and resulted pectin was washed with ethanol, and dried in vacuo. Mitsumata pectin was prepared by the same procedure.

Ganpi pectin was used as the substrate of ME. The reaction media contained 30 ml of 1 per cent Ganpi pectin solution, 20 ml of mixed solution of enzyme and water, 10 ml of phosphate buffer, and 0.6 ml of toluol. Four reactions were carried out at 37°C, and three reaction media contained 5, 10, 20 ml of ME solution respectively, and the fourth was a blank test. After the reactions completed, 10 ml of media was taken into a beaker, and residual pectin determined. As shown in Table III, ME was found to hydrolyze pectin obtained from Ganpi.

TABLE III. ACTION OF MACERATING ENZYME ON GANPI PECTIN

Volume of enzyme soln. employed ml	Ratio of decrease of pectin %			
	2 hrs	4 hrs	8 hrs	24 hrs
5	0	3.3	7.2	49.9
10	1.5	8.0	34.2	64.6
20	9.9	22.8	49.8	66.4

Citrus pectin was prepared from commercial pectin. Pectin was also extracted from segments of Natsudaidai orange with water of which the pH was adjusted to 2.2 by the addition of citric acid, and was kept at 100°C, for 30 min.

The amount of methoxyl radical was determined by Zeisel's method, and the results are shown in Table IV.

The activity of ME on various pectins was observed by the decrease in the amount of pectin caused by the action of enzyme for 24 hrs. In the other experiment, the activity was determined by the increase in reducing value and by the decrease in viscosity as described in Table IV.

From these findings it was found that ME is able to hydrolyze Ganpi and Mitsumata pectin more rapidly than citrus pectin. Natsudaidai pectin was decomposed in almost the same rate as citrus pectin. In the other experiment, pectin which was prepared from Natsudaidai by ammonium oxalate was attacked by ME in almost the same extent as Mitsumata or Ganpi pectin.

From these results, it was found that ME was active to pectin obtained from various plant materials.

IV. Actions of Polygalacturonase and Macerating Enzyme on Pectic Acid.

Pectic acid was prepared from citrus pectin by the method of Norris and Resch[12]. The reaction media contained 15 ml of 1 per cent of pectic acid solution, 5 ml of phosphate buffer and 10 ml of PG solution prepared as mentioned above. The reaction was carried out at 37°C, for 24 hrs. After the reaction was completed, the residual pectic acid was determined, and the ratio of pectic acid decrement represented as the activity of PG.

TABLE IV. AMOUNT OF METHOXYL CONTENT IN VARIOUS PECTIN AND ACTION OF MACERATING ENZYME ON PECTIN

Sources of pectin	Methoxyl content %	Action of macerating enzyme		
		Decrease of pectin after 24 hrs' reaction	Increase of reducing value after 24 hrs' reaction	Decrease of viscosity after 2 hrs' reaction *A*
		%	ml	%
Citrus	8.94	17.2	—	—
Natsudaidai orange	11.15	14.0	0.03	6.7
Ganpi	3.33	62.0	0.18	58.7
Mitsumata	3.04	53.0	0.15	44.1

The increase of reducing value was represented as 0.02 N iodine solution consumed per 2 ml of reaction medium.

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[12] F. W. Norris and C. F. Resch, *Biochem. J.*, **31**, 1945 (1937).

The action of ME on the same substrate was performed by the same procedure, and the actions of PG and ME on the barks of Ganpi was also investigated by the reaction media which contained a piece of Ganpi, 0.5 ml of phosphate buffer and an amount of 1 to 3 ml of enzyme solution. These results are shown in Table V. A small amount of hydrolysis occurred after 24 hours' action of ME on pectic acid, and no maceration of the barks was observed in the action of PG.

TABLE V. ACTIONS OF ME AND PG ON PECTIC ACID AND ON BARKS OF GANPI

	Ratio of decrease of pectic acid %	Separation of fibres (1.0) ml	(2.0) ml	(3.0) ml
PG	66.0	–	–	–
ME	8.2	+++	++++	+++++

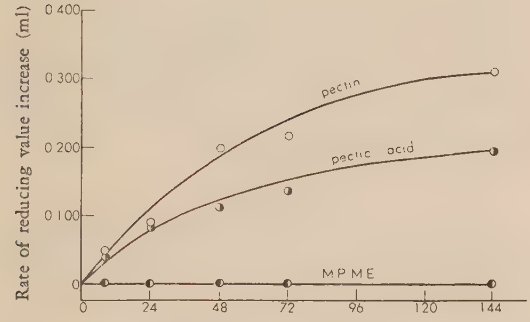

FIG. 2. Reaction Cause of Macerating Enzyme on Mitsumata Pectin, Pectic Acid and MPME.
Determined by reducing value.

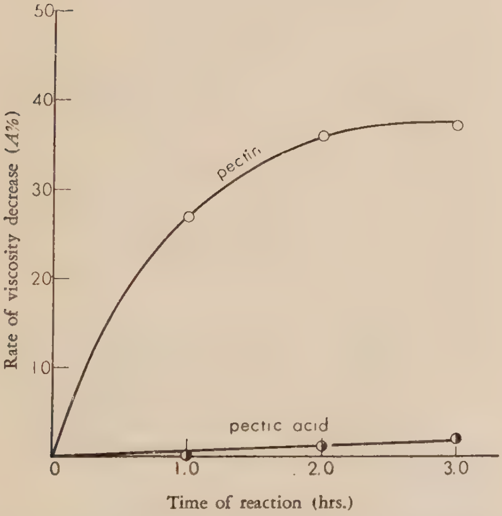

FIG. 3. Action of Macerating Enzyme on Mitsumata Pectin and Pectic Acid.
Determined by decrease of viscosity.

In the other experiment, Expl. V, the action of ME on pectic acid was determined by the increase in the reducing value and the decrease in viscosity in comparison with the action of ME on Mitsumata pectin, the results being shown in Fig. 2 and Fig. 3. A remarkable decrease in viscosity was observed in the reaction medium which contained pectin, but the extent of decrease A was very small in the case of pectic acid throughout 3 hours' reaction. The increase in reducing value revealed a very small extent of hydrolysis in both cases of pectin and pectic acid.

V. Action of Macerating Enzyme on Methylglycoside of Polygalacturonic Methyl Ester (MPME).

Mitsumata pectin was converted into pectic acid, and MPME was prepared from pectic acid by the method of Morell, Baur and Link[13,14].

Actions of ME on Mitsumata pectin, pectic acid and MPME were examined by the use of the reaction media containing 15 ml of 1 per cent MPME, pectin or pectic acid solution, 5 ml of phosphate buffer and 10 ml of ME solution. A blank test for each reaction was also performed. The increase of reducing value in 2 ml of reaction media was determined by a modified Willstätter, Schudel method. As shown in Fig. 2, ME was found to be almost inactive on MPME throughout the 6 days incubation. And thus, it was demonstrated that ME could not attack glycosidic linkage of the esterified galacturonic acid residues.

DISCUSSION

(1) The author has already reported that ME and PG could be separated by the use of Amberlite IRC–50 and the decrease in pectin was found to be only 6.3 per cent that of middle lamella pectin when the separated ME acted on the barks of Ganpi. In the previous paper[6], the author described that ME could be obtained by a simple procedure using Duolite CS–101 and is more active than the enzyme prepared by the chromatography of

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13) S. Morell, L. Baur and K. P. Link, *J. Biol. Chem.*, **105**, 1 (1934).

14) S. Morell, K. P. Link, *ibid.*, **100**, 385 (1933).

Amberlite IRC–50.

In the present paper, ME, thus separated, acted on the barks of Ganpi until the decrease of pectin proceeded from 8.9 to 22.0 per cent of pectin contained in the barks.

ME also revealed hydrolysis of the pectin which was extracted from barks of Ganpi with ammonium oxalate.

It was found that pectin in the middle lamella is not eluted as free pectin, but the hydrolysis of the pectin is caused by the enzyme, and thus maceration of the plant tissue occurs.

(2) The hydrolysis of Ganpi pectin with ME was found to take place more easily than pectic acid which was prepared from citrus and Ganpi pectins. On the other hand, PG which was separated by the procedure mentioned in the previous paper could easily hydrolyze pectic acid, but could not macerate the barks of Ganpi etc. From the results of these experiments, it is considered that ME is a sort of pectin glycosidase according to Schubert's definition[15], differing from PG which hydrolyzes pectic acid more rapidly than pectinic acid.

(3) It has been reported that *Byssochlamys fulva* produced the enzymes which presented maceration action[16]. However, it is also reported that PG and PE are excreted by *B. fulva*[17]. Lineweaver and Jansen[18] suggested that the combined action of PG and PE might give rise to maceration of the plant tissue. As mentioned above, the separated ME showed no PE action on Mitsumata and citrus pectins, and it was thus demonstrated that ME obtained in the present experiment revealed a single maceration of plant tissues without any combined action of PG and PE.

(4) Seegmiller and Jansen[2] studied on PMG which attacked citrus pectin, and Roboz, Barratt and Tatum[8] reported on PDP which was related to the hydrolysis of pectic acid and citrus pectin. Some natures of ME obtained in the present experiment were found to be similar to PMG and PDP, since ME hydrolyzed pectin more rapidly than pectic acid, and optimum pH of ME was found to be 6.0. However, ME is differs from PMG and PDP in its action on citrus and Natsudaidai pectins, for which ME revealed hydrolysis in a lower rate than Ganpi or Mitsumata pectin. In the same respect, ME seems to be different from yeast PG[19] and *Asp. foetidus* PG[20]. Furthermore, ME differed distinctly from PMG and PDP in its action on MPME, because ME was almost inactive on MPME, while PMG and PDP were exceedingly active, as verified in the studies cited above.

SUMMARY

1) Macerating enzyme (ME) was separated as a single protein from cultural broth of *Cl. felsineum* var. *sikokianum* by column-chromatography using Duolite CS–101.

2) ME was obtained as a single enzyme, and it was able to macerate the barks of Ganpi and Mitsumata, tubers of potato and sweet potato, roots of radish and carrot, and segments of orange.

3) It was found that ME attacked the middle lamella pectin of the plant materials, and maceration action was resulted by hydrolysis of the pectin.

4) ME could also hydrolyze pectin which was extracted from various plant materials with ammonium oxalate solution.

5) ME hydrolyzed Mitsumata pectin more rapidly than pectic acid.

Acknowledgment. The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. for his guidance and encouragement. He is also indebted to Mr. Y. Anabuki of this laboratory for his assistance in this experiment.

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[15] E. Schubert, *Helv. chim. acta*, **37**, 691 (1954).

[16] G. H. Beavan and F. Brown, *Biochem. J.*, **45**, 221 (1949).

[17] W. W. Reid, *Nature*, **166**, 76 (1950).

[18] H. Lineweaver and E. F. Jansen, "Pectic Enzymes" Advances in Enzymology (F. F. Nord) Vol. XI, pp. 228 (1951).

[19] B. S. Luh and H. J. Phaff, *Arch. Biochem. Biophysic.*, **51**, 102 (1954).

[20] A. Ayres, J. Dingle, A. Phipps, W. W. Reid and G. L. Solomons, *Nature*, **170**, 834 (1952).

Microbiological Oxidations of Reichstein's Compound S by *Corticium solani*

Sir:

It has been found by the authors that some fungi of *Corticium*, especially *C. sasakii*, the fungus of the sheath spot disease of rice plants, hydroxylated Reichstein's Compound S to hydrocortisone, epi-hydrocortisone[1] and 19-hydroxy-Compound S[2]. We wish to report here on a different type of the microbiological oxidations of Reichstein's Compound S by *Corticium solani* (*Corticium vagum*), a root-rot fungus of various plants, which is not the same microorganism as *Corticium sasakii* but is closely related to it. Six hundred mg. of Reichstein's Compound S (I) was incubated with a 72-hour growth culture of *Corticium solani* IFO, No. 6251 in 1 l. of a synthetic medium (Table) for 16 hours on a reciprocating shaker.

After extracted with ethyl acetate and concentrated, the conversion products were found to be composed of three major substances on a paper chromatogram, and then each of them was separated by chromatography on Florisil using a methylene chloride—acetone system.

TABLE

NH_4NO_3	0.2%
K_2HPO_4	0.1%
$FeCl_2 \cdot 4\,H_2O$	0.05%
KCl	0.05%
$MgSO_4 \cdot 7\,H_2O$	0.05%
Soluble starch	3%
Vitamin solution*	1%
pH	ca 6.5

* Vitamin solution

Biotin	0.2 mg
Ca-pantothenate	40 mg
Folic acid	0.2 mg
Inositol	200 mg
Niacin	40 mg
PABA	20 mg
B_6HCl	40 mg
FMN	20 mg
B_1HCl	40 mg/1000 ml

Methylene chloride—acetone (200 : 15) fractions afforded a crystalline product: m. p. 240–241°, $[\alpha]_D^{19}=+75°$ (c, 1.0% in chloroform), $\lambda_{max}^{EtOH}=244\ m\mu$ ($\epsilon=17{,}700$) (*Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.25; H, 8.14. Found: C, 72.94; H, 8.25). These constants quite agreed with $\Delta^{1,4}$-pregnadiene-17α, 21-diol-3, 20-dione (Δ^1-Reichstein's Compound S)[3] (II). Comparison of the infrared spectrum of the compound with that of an authentic sample as well as the mixed melting point of them confirmed the identity.

The second product was separated from methylene chloride—acetone (200 : 30—200 : 100) fractions and recrystallized from acetone; m. p. 233–234°, $[\alpha]_D^{20}=+54°$ (c, 0.5% in dioxane), $\lambda_{max}^{EtOH}=236\ m\mu$ ($\epsilon=15{,}600$) (*Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.19; H, 8.24). By acetylation, a diacetate was given: m. p. 190–191° (uncorrected), $[\alpha]_D^{21}=+63°$ (c, 2.0% in $CHCl_3$), $\lambda_{max}^{EtOH}=235\ m\mu$. A monoacetate had the following constants: m. p. 258–260°, $[\alpha]_D^{21}=+78°$ (c, 1.0% in dioxane), $\lambda_{max}^{EtOH}=236.5\ m\mu$ ($\epsilon=15{,}000$). The chromic oxidation product, a yellow crystalline substance: m. p. 217–218° (uncorrected), $[\alpha]_D^{21}=+49°$ (c, 1.0% in acetone), $\lambda_{max}^{EtOH}=248\ m\mu$. These data of the second compound and its derivatives were identical with those of 6β-hydroxy-Compound S (Δ^4-pregnene-6β, 17α, 21-triol-3, 20-dione) (III) and its derivatives in the literature by Peterson et al.[4]. The third compound (IV) is now under investigation.

From the above observations it was con-

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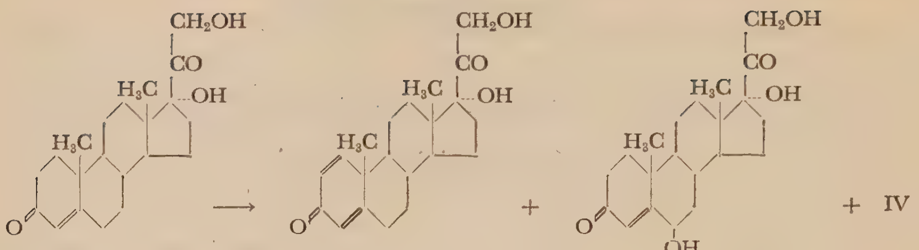
1) T. Hasegawa et al., *Bull. Agr. Chem. Soc. Japan*, **21**, 390 (1957).

2) M. Nishikawa et H. Hagiwara, *Chem. Pharm. Bull.*, **6**, 226 (1958).

3) A. Nobile et al., *J. Am. Chem. Soc.*, **72**, 4184 (1955).

4) D. H. Peterson et al., *J. Am. Chem. Soc.*, **75**, 412 (1953).



I II III

cluded that *Corticium solani* IFO, No. 6251 catalyzed the reactions of the 1,2-dehydrogenation and the 6β-hydroxylation of Reichstein's Compound S.

The authors wish to express their sincerest thanks to Dr. K. Sato, Institute for Fermentation, and to Dr. T. Matsukawa, Research Laboratories attached to Takeda Pharm. Ind., for their guidance and encouragement throughout this work, and also to Dr. M. Nishikawa and Mr. H. Hagiwara for their advice.

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Received December 19, 1958

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Formation of Native Starch-like Granules of Amylodextrin in Vitro

Sir:

A specifical kind of sweet potato starch syrup has recently been manufactured as an additional carbohydrate in the new process of Saké-brewing. In some cases, a white flocky precipitate was formed in these starch syrups. This precipitate was centrifuged and washed repeatedly with a small amount of cold water. It consisted of an irregular small mass without birefringence under the microscope. However, they showed a very sharp 'A'-type X-ray diffractometer curve as shown in Fig. 1–II. The mean degree of polymerization of the precipitate measured by the alkaline ferricyanide method[1] was 12.0 and its specific rotation $[\alpha]_D^{20}$ was +187.5°. The wave length of max. absorption spectrum of its iodine complex (λ_{max}) was 460 mμ. It was easily soluble in warm water and completely hydrolyzed by crystalline sweet potato β-amylase. All these properties clearly showed that this part of the amylodextrin was entirely composed of a short chain amylose component.

This linear amlodextrin was recrystallized from various solvents, and regular small spheres with birefrigence exactly resembling native starch granules were obtained.

Recrystallization was carried out as follows: The amylodextrin was suspended in ten times or more of aqueous methanol-*n*-propanol solution (methanol: *n*-propanol: water=3:3:4, by volume) and the suspension was heated in a boiling water bath. A faint opaque solution was obtained. After cooling for several hours at room temperature this was kept in a refrigerator for several days. About 50% of the original amount was precipitated as regular

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[1] S. Nussenbaum, W. Z. Hassid, *Anal. Chem.*, **24**, 501 (1952).

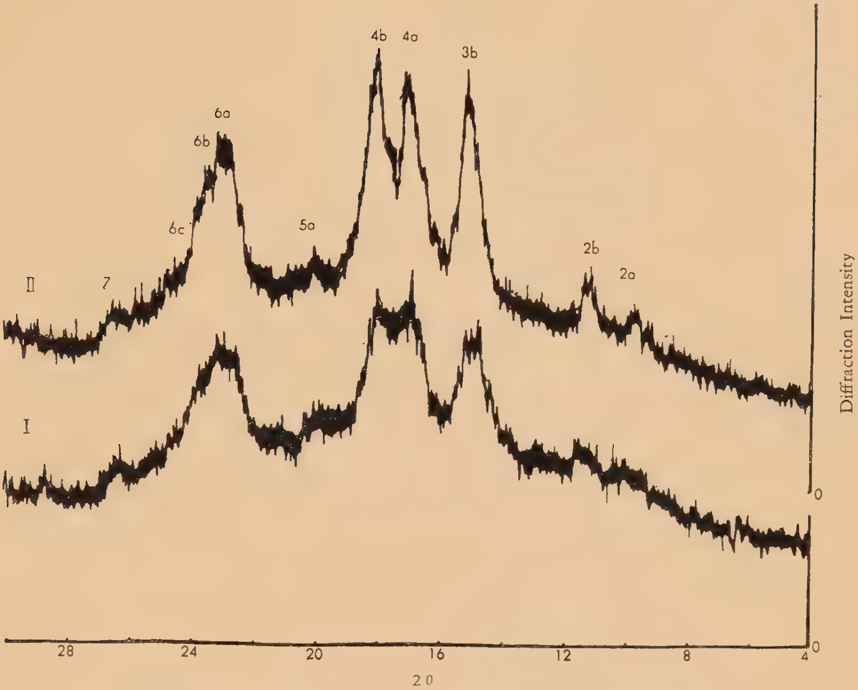

FIG. 1. X-ray Diffractometer Curves of Native Corn Starch and Amylodextrin Granules Crystallized from Aqueous Methanol-*n*-propanol Solution.

I: corn starch, II: amylodextrin.

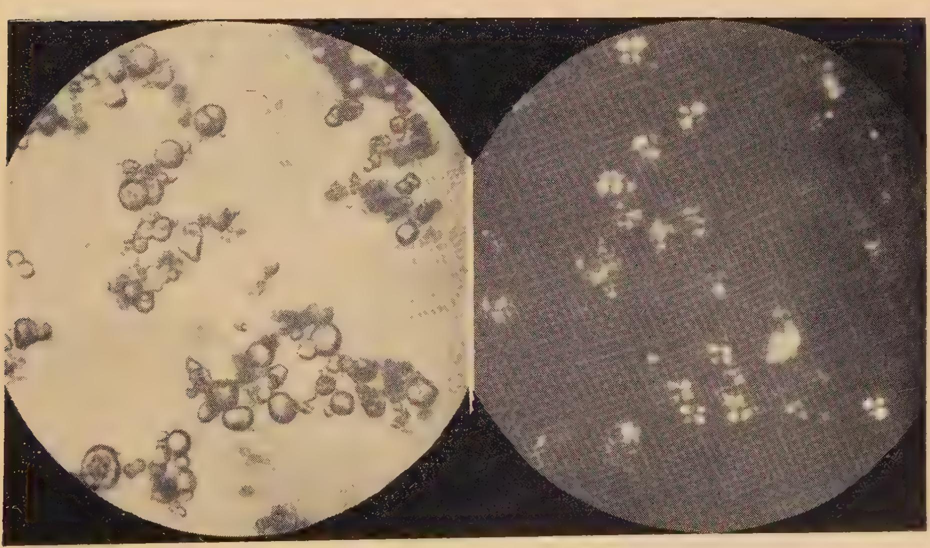

FIG. 2. Photomicrographs of the Same Artificial Amylodextrin Granules in Fig. 1 under Usual (left) and Polarized (right) Light. ×814

small granules comparable with native starch granules in both shape and optical property (Fig. 2). The granules thus obtained gave 'A'-type X-ray diffractometer curve, the same as native cereal starches (Fig. 1). But the intensity of the diffraction lines was about twice of those of native starches showing higher crystallinity than native starches. The mean degree of polymerization of this material was 15.0, $[\alpha]_D^{20}$ was $+196°$, and λ_{max} was 485 mμ.

When the amylodextrin was recrystallized from an aqueous solution (ca. 33%), anisotropic amylodextrin granules which showed 'B'-type X-ray diffractometer curve the same as native tuber starches were obtained. The mean degree of polymerization of this material was 14.9, $[\alpha]_D^{20}$ was $+195°$, and λ_{max} was 480 mμ.

X-ray diffractometer curves were obtained by a 'Norelco' X-ray diffractometer using a nickel-filtered copper radiation.

From these results, it was shown that the artificial amylodextrin granules might have the same molecular arrangements and micelle orientations as native starch granules.

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[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2, p. 140~141, 1959]

Terpenoids. Part VI.

Further Investigation on the Constitution of Occidentalol

Sir:

The constitution of the sesquiterpene alcohol, occidentalol (I), $C_{15}H_{24}O$, m.p. 95°C, isolated from *Thuja occidentalis* L. was investigated and proposed in the previous paper[1].

Further investigation was carried out to confirm the positions of the conjugated ethylenic linkages and that of the angular methyl group in the proposed formula.

Occidentalol was reacted with dimethyl acetylene-dicarboxylate at 200°C for 2 hours. The product was then subjected to pyrolysis to give the following fractions: (1) b.p. 75–90°C/10 mm, (2) b.p. 130–140°C/10 mm, (3) b.p. 155–160°C/10mm. On dehydrogenation with 5% palladium charcoal, fraction (1) gave *p*-cymene (II), b.p. 172–174°C, n_D^{14} 1.4875, which was identified by its infrared spectrum and conversion into *p*-hydroxyisopropylbenzoic acid, m.p. and mixed m.p. 157°C, with potassium permanganate. Fraction (2) was unreacted occidentalol and the third fraction was saponified with 10% methanolic potassium hydroxide solution to afford 3-methylphthalic acid (III), m.p. 153–154°C (*Anal.* Found: C, 59.47; H, 4.24. Calcd. for $C_9H_8O_4$: C, 60.00; H, 4.48). Furthermore the latter was converted into 3-methylphthalic anhydride, m.p. 116–117°C (*Anal.* Found: C, 66.14; H, 3.19. Calcd. for $C_9H_6O_3$: C, 66.67; H, 3.73).

These results leave no doubt that occidentalol is correctly represented by (I), and thus the constitution of occidentalol has been established.

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Received February 4, 1959

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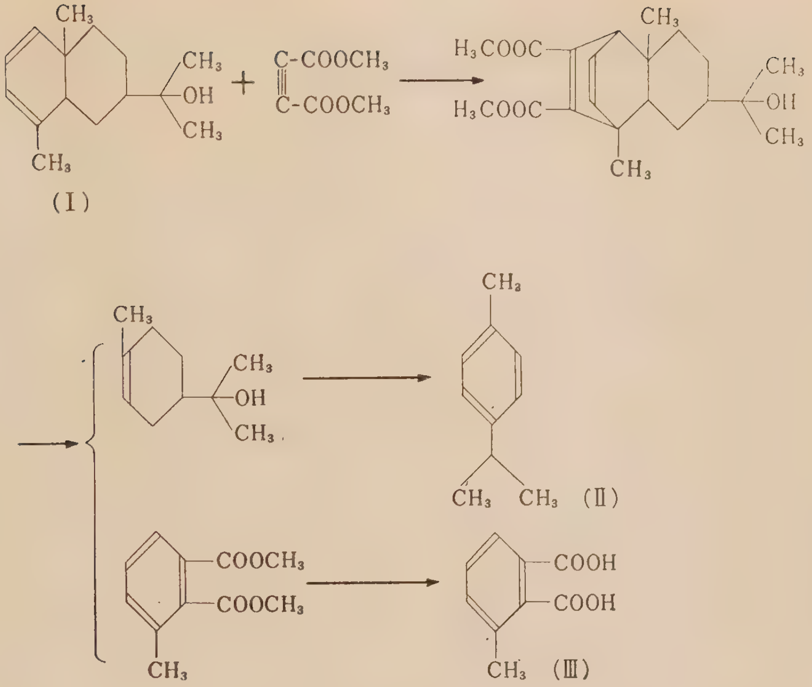
[1] T. Nakatsuka and Y. Hirose, This Bulletin, **20**, 215 (1956).



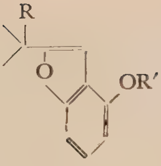
[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2, p. 141～142, 1959]

Synthesen und Konfigurationsermittlung in der Rotenoid-Reihe,

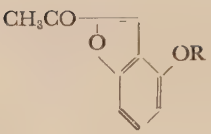
IX. Synthese des Roteols und Hydroxyroteols

Im Verlaufe von Untersuchungen über Rotenoiden haben wir das Roteol (I, $R=R'=H$) und Hydroxyroteol (I, $R=OH$, $R'=H$) synthetisiert.

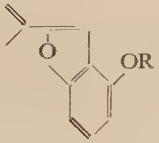
2-Acetyl-4-hydroxy-cumaron (II, $R=H$) reagiert nicht mit Methylmagnesiumjodid wegen seiner Unlöslichkeit in Äther. Das genannte Cumaron liefert bei der Umsetzung mit Benzylbromid in Gegenwart von Kariumcarbonat in Aceton 2-Acetyl-4-benzyloxy-cumaron (II, $R=CH_2C_6H_5$), welches anschliessend die Grignard Reaktion erleidet werden konnte. Nach einer gewöhnlichen Aufarbeitung haben wir 2-(α-Hydroxy-isopropyl)-4-benzyloxy-cumaron (I, $R=OH$, $R'=CH_2C_6H_5$) und 2-iso-Propenyl-4-benzyloxy-cumaron (III, $R=CH_2C_6H_5$) erhalten. Von denen geht das erste bei der katalytische Hydrierung in Gegenwart von Palladium-Kohle in Äthanol unter Aufnahme von 1 Mol. Wasserstoff in das Hydroxyroteol (I, $R=OH$, $R'=H$) über. Bei einer analogen Hydrierung nimmt das andere 2 Mol. Wasser-



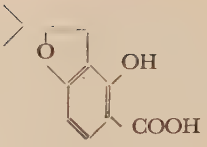
I



II



III



IV

I, R=R′=H	59-60°C	$C_{11}H_{12}O_2$	Ber.	C 74.84	H 6.86%
			Gef.	74.64	6.73%
I, R=OH, R′=H	160-161	$C_{11}H_{12}O_3$	Ber.	C 68.73	H 6.29%
			Gef.	68.64	6.26%
I, R=OH, R′=$CH_2C_6H_5$	Glas	$C_{18}H_{18}O_3$			
II, R=$CH_2C_6H_5$	113-114	$C_{17}H_{14}O_3$	Ber.	C 76.67	H 5.30%
			Gef.	76.49	5.39%
II, R=$COCH_3$	117-118	$C_{12}H_{10}O_4$	Ber.	C 66.05	H 4.62%
			Gef.	66.01	4.85%
III, R=$CH_2C_6H_5$	82-83	$C_{18}H_{16}O_2$	Ber.	C 81.79	H 6.10%
			Gef.	81.87	6.18%

stoff und liefert in fast quantitativer Ausbeute ein bei 142–3°C/10mm. siedendes Öl (n_{D}^{18} 1.5635), welches spontan kristallisiert. Schmp. ca 45°C. Nach einem Umkristallisieren aus Petroläther schmolz die weissen feinen Kristalle bei 59 bis 60°C (gut Übereinstimmung mit Angaben des Schrifttums[1]). Das Roteol vom Schmp. 60°C ist erst von Takei[1] durch eine Decarboxylierung der Rotensäure (IV), eines Abbauprodukts des Rotenons, erhalten worden. Reichstein[2] hat das Roteol in einem komplizierten Weg synthetisch dargestellt. Der schweizere Autor hat den Schmp. 37~9°C (nach Umkristallisieren aus Pentan) der synthetischen Probe mitgeteilt.

Durch die Einwirkung von Pyridin tritt das Cumaron (II, R=H) mit Acetanhydrid in 2-Acetyl-4-acetoxy-cumaron (II, R=$COCH_3$) zusammen, welches durch Umsetzung mit Methylmagnesiumjodid das Hydroxyroteol in nur mässiger Ausbeute liefert. Daneben wird das Ausgangsmaterial (II, R=H) zurückerhalten; ihre Abscheidung ist schwer zugänglich.

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Eingegangen am 5, Februar, 1959

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[1] S. Takei, *Ber.*, **61**, 1003 (1928).

[2] T. Reichstein und R. Hirt, *Helv.*, **16**, 121-9 (1933).

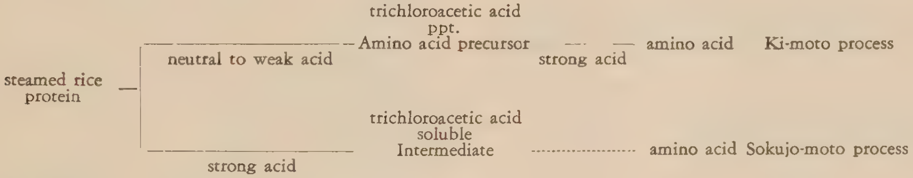
Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 33, No. 1 (1959)

The Formation of Amino Acids in Ki-moto. Part III. On the Role of Lactic Acid in Moto Making. (p. *1*~*6*)

By Hiroichi AKIYAMA
(*Brewing Experiment Station, Tax Administration Agency*)

From previous papers the author succeeded in making moto mashes like Yamahai-moto, one of Ki-moto, in large scale, controlled lactic acid bacteria by penicillin and added lactic acid in parts, and by using these moto fermented moromi mashes and brewed fine saké.

The methods of addition of lactic acid to moto mash and decomposition of N-compounds in it were studied. The presumable process of decomposition of N-compounds in moto would be as follows:



So as in the above scheme, the decomposition of N-compounds in moto would be controlled by velocity of acidification and concentration of lactic acid. When the proteolytic action was proceeded in from neutral to weak acidic, moto would be rich in amino acids like Ki-moto, however, in strong acid during moto making, moto would be poor in amino acids like Sokujo-moto.

From these reasons the author added lactic acid in part during moto making, and could improved the contents of amino acids in Sokujo-moto like Yamahai-moto.

Studies on Bacterial Proteinases. Part XI. Microbiological Properties and Culture Condition of An Alkaline Proteinase Producing-*Bacillus subtilis*. (p. *6*~*9*)

By Juichiro FUKUMOTO, Takehiko YAMAMOTO and Kazuhiro ICHIKAWA
(*Institute of Polytechnics, Osaka City University*)

An investigation was undertaken on some microbiological properties and culture condition of a strain of *Bac. Subtilis* which produced a proteinase active at pH 9.5~10.5 (alkaline proteinase). The bacteria was characteristic in forming a pellicle of faint reddish color on a soybean cake extract medium. The greatest enzyme activity was obtained when the bacteria was cultured submergedly on a medium containing 10% soybean cake extract, 3% dextrin and 0.2% sodium citrate or when grown on a medium consisting of each 3 parts of wheat bran and soybean cake meal and 4 parts of rice bran, for two days at 35°C. The addition of citrate in small amounts to liquid media always resulted in good production of the alkaline proteinase. The pH value of media between 7.0 to 8.0 was most favorable for enzyme production and, at pHs lower than it, the bacteria secreted another type of proteinase together with the alkaline proteinase, the former being most active in hydrolyzing gelatin at pH 7.0–7.4 (neutral proteinase). At bran solid culture, increasing the amount of rice bran to a ratio greater than that described above gave little effect in the yield of enzyme activity determined at a certain pH value, but in this case it was found to occure a decrease in enzyme activity of the alkaline proteinase while an increase in that of the neutral proteinase.

Studies on Bacterial Proteinases. Part XII. Crystallization and Some Properties of An Alkaline Proteinase of *Bacillus subtilis*. (p. *9*~*13*)

By Juichiro FUKUMOTO, Takehiko YAMAMOTO and Kazuhiro ICHIKAWA
(*Institute of Polytechnics, Osaka City University*)

The alkaline proteinase of a strain of *Bac. subtilis* previously reported was obtained in a crystalline state by a method similar to that carried out in a crystallization of the neutral proteinase which was secreted by another strain of the same bacterial species (This Journal, **32**, 230 (1958)). In this case, however, fractionation of enzyme with alcohol was performed at a stage of purification and two proteinase preparations were obtained: one being precipitated at concentrations of 20 to 40%, and the other at 50 to 70%, of alcohol. However, both of them acted best at pH 10.5 when tested by a modification of the Folin's method and there was no remarkable difference in enzymatic properties between them. The alkaline proteinase was most stable at pH 9.0 to 9.5 in an incubation test at 30°C for 24 hrs. The enzyme revealed to hydrolyze casein to a degree considerably greater than that with the neutral proteinase. Enzyme activity of the proteinase was not affected by the addition of Versene as found with "Subtilisin" by Güntelberg and Ottesen (*Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **29**, 36 (1954)). But the enzyme was completely inhibited by the addition of a certain protein in potato, known as a protease inhibitor. Alkaline proteinase of *Bac. subtilis* was found to be easily distinguished from the neutral proteinase on the two points of the enzymatic properties described fiinally.

On the Halophilic Character of Shoyu-Yeast. Part IV. Effects of Inhibitors on the Halophilic Behaviour of the Yeast. (1) (p. *13~18*)

By Masahiro SATO and Teijiro UEMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

Effects of DL-ethionine and 8-azaguanine on the yeast halophilism were studied with soy-sauce yeasts, *Zygosaccharomyces major* st. *Kikkoman* and *Torulopsis* sp. (a choline requiring strain).

It was observed with major strain that the presence of 10% (w/v) NaCl in the defined media markedly enhanced the rates of inhibitory effects of ethionine on the yeast growth in the circumstances deficient in either of inositol, choline and S-containing amino acids. The inhibition by ethionine was reversed by the addition of methionine. Mixture of inositol and choline was also partially effective. Neither of both these vitamins showed, if at all, reversary effects, when singly supplied in the presence of NaCl. However, the antagonistic effect of choline against the inhibition by ethionine was well observable in the absence of NaCl, whereas in contrast inositol did not show such a effect in either its presence or absence. The findings as described above were also the case employing the choline requiring strain as the test organism and in this case the effect of choline was more distinctly observed.

8-Azaguanine when supplied to the cultures of major strain markedly inhibited the yeast growth in the presence of 10% NaCl whereas slightly in its absence.

When tested with non-halophilic yeasts such as *Sacch. sake*, *Sacch. cerevisiae* and *Zygosacch. barkeri* the antagonistic effects of inositol, choline or methionine respectively on them were also demonstrated in the absence of NaCl.

Finally the experiments on the assimilation of various kinds of nitrogen sourced by *Zygosacch. major* revealed the fact that this strain could not assimilate urea as the sole source of nitrogen in the 10% NaCl-containing medium.

On the Halophilic Character of Shoyu-Yeast. Part V. Effects of Inhibitors on the Halophilic Behaviour of the Yeast. (2) (p. *18~22*)

By Masahiro SATO and Teijiro UEMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

The inhibitory effects of 8-azaguanine on the yeast halophilism were more precisely examined with a halophilic strain of soy-sauce yeast, *Zygosacch. major* st. *Kikkoman*.

8-Azaguanine when supplied at the concentration as low as below 100 γ per ml. gave an increased inhibitory effect on the yeast growth if NaCl was to some extent added to the medium.

Addition of equimolar guanine well antagonized against the inhibitory effect of 8-azaguanine and this was also the case in the supply with S-containing amino acids such as cystine and methionine. The antagonistic effect of these amino acids was easily demonstrable at the higher concentration of 8-azaguanine (200 γ per ml.) but hardly at its lower concentration (100 γ per ml.).

From the results obtained it was assumably mentioned that the halophilic mechanisms of the soy-sauce yeast seemed to be closely related to the nucleic acid metabolism in the yeast cell and the effective vitamins and amino acids such as inositol, choline and S-containing amino acids played a role in participating in nucleic acid metabolism of the yeast, inducing its halophilic character.

Studies on the Requirements of Amino Acid and Vitamins. Part. VI. The Effect of Metals on the Growth of *Sacch. saké*. (p. *23~27*)

By Masahiro TAKAHASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The author examined the effect of Fe, Mn, Co, Cu, Ni and Zn added to the medium on the growth of *Saccharomyces saké*. It was discovered that copper ion could recover the inhibitory action of histidine on the growth. The optimum concentration of copper ion was 10^{-5}M.

Determination of Phosphorus, Radiophosphorus and Chromic Oxide in Feces with a Solution obtained by Perchloric Acid Oxidation. On the Methods for the Study of Digestion and Absorption Part I. (p. *27～31*)

By Ichiro YOSHIHARA, Akio SUGISAKI
(*Faculty of Agriculture, University of Niigata*)

For the study of absorption and excretion of phosphorus, it is desirable to use chromic oxide as an index material in addition to radiophosphorus. Methods for the estimation of phosphorus and for the assay of radiophosphorus by the use of perchloric acid have been established, but there is no evidence that they are also applicable to the feces containing chromic oxide.

About 1.2 g of feces of a rabbit fed on a diet, consisting of equal proportion of wheat bran, commercial mixed feed for dairy cows and powered hay, and 46–95 mg. of chromic oxide were placed in Kjeldahl digestion flask and digested in 20 ml. of perchloric acid (60%). The feces only were digested in the same way. Using these solutions, effects of the presence of chromium on the estimation of phosphorus and on the assay of added radiophosphorus were studied. Moreover the oxidation of chromic oxide was studied.

The results obtained were summarized as follows: Phosphorus, radiophosphorus and chromic oxide in feces were determined successfully with a solution obtained by perchloric acid oxidation of the feces and consequently the method for determination was decided.

Elson Morgan Reaction of N-Substituted-isoglucosamines. (p. *31～34*)

By Toshio OHNISHI and Yoshiyuki INOUE
(*Biochemical Laboratory, College of Agriculture, Kyoto University*)

In the Elson Morgan test some N-substituted-isoglucosamines gave a red colour. The positions of the absorption maxima were at 530 mμ and 560–570 mμ. Hexsosamines gave an absorption at 530 mμ. N-(aminoacid)-Isoglucosamines and N-arylisoglucosamines showed a different behaviour in Elson Morgan test from N-arylglucosylamines, which gave no absorption at 530 mμ.

It is suggested that these colour reactions of N-substituted-isoglucosamines and hexosamines is based on the similar mechanism.

Heterogeneity of α-Casein and Rennin Coagulation. Part I. (Studies on Milk Coagulating Enzymes. VIII) (p. *35～38*)

By Tomokichi TSUGO and Kunio YAMAUCHI
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

α_R-Fraction (α_R-casein) and κ-fraction (crude κ-casein) were separated from Warner's α-casein and it was demonstrated that the mobility of the former was not affected by rennin but that of the later was decreased after rennin action by means of Tiselius electrophoresis in paosphate-NaCl buffer (pH 7.3; μ=0.1). This was confirmed by electrophoretic experiment on mixtures (mixed at low temperature previous to electrophoresis) of both fractions treated or not treated with rennin. The disagreement of patterns between rennin-treated α-casein and mixture of two fractions treated with rennin respectively was pointed out and reason for this was discussed. In unseparated α-casein interaction between both fractions probably remains even after the rennin action.

Heterogeneity of α-Casein and Rennin Coagulation. Part II. (Studies on Milk Coagulating Enzymes. IX) (p. *38～42*)

By Kunio YAMAUCHI and Tomokichi TSUGO
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

α_R-fraction (α_R-casein) and κ-fraction (crude κ-casein) were separated from Warner's α-casein and rennin actions on both fractions were compared. The former was coagulated with adding $CaCl_2$ (0.05 M), while the later remained clear solution. After rennin action κ-fraction became clottable in the presence of $CaCl_2$ at 35°C but not at 0°C. Stabilizing action of the later for the former was lost by rennin. κ-fraction liberated non protein nitrogen (12% trichloroacetic acid soluble N) specifically (about 8% of total N) by rennin, while α_R-fraction was scarecely decomposed by it. κ-fraction became opalescent in calcium free solution (0.085 M NaCl) by rennin and this opalescence was increased by heating (80°C, 5 minutes). α_R-fraction showed no change by rennin. κ-fraction became irreversibly denaturable by urea (6.66 M) after rennin action; the precipitate appeared in rennin treated κ-fraction after removal of urea by dialysis.

It is assumed that rennin action is specific for κ-

casein and that κ-casein translates into the structural configuration which is easily denatured by heating or urea treatment.

Studies on she Utilization of Ion Exchange Process in Sugar Refining. Part VI. On the Restoring Procedures for the Color Contamination of Strongly Basic Anion Exchange Resins. (p. *42～45*)

By Susumu IWASHINA

(*Research Institute of Meiji Sugar Mfg. Co. Ltd., Kawasaki, Japan*)

There are two methods directly and indirectly used to cope with the color contamination of anion exchange resin (AER). The former is the restoration and the latter the pretreatment of the influent liquor.

This report relates to the restoring procedures, which is generally devided into two methods: the first method of using the mineral acid and its salts, and the second one of using the solution of a water-soluble hypohalite—particularly a hypochlorite—and the other solution of a mild oxidizing agents.

In all of the methods, HCl and NaClO are usually used. In connection with the effect of the treatment, the external changes have been recorded in the microscopic color film, and the variable development of ion exchange capacities has been examined.

The test pieces are 4 species of previously reported* color contaminated of the $R\equiv NX$ type AER which have developed for 20 cycles, Type I—Porous, Type I—Porous, Type II—Porous and Type II—None Porous AER products sold on market.

In the first method, the capacity drop caused by the contamination was effectively recovered. In the second method, howerver, affected by the oxidization of functional group and polymer chain of the resin, the exchange capacity decreased. Thus the socalled basicity drop as well as the decrease in physical strength of the resin matrix were observed. In reference to the apparent change, the NaClO method disclosed a significant decrease in coloring. It was difficult to judge the effect upon color contaminated of the resin by appearance alone.

It has been proved upon that the chemical and physical characteristics of the resin in the restoring procedure, and the contamination should be considered to yield the effect upon color contaminated of the resins.

* S. Iwashina: *J. Agr. Chem. Soc. Japan*, **32**, 882 (1958).

Studies on the Classification of *Streptomyces griseus* Group. Part VI. Classification by the Serological Properties (p. *45～48*)

By Yujiro HARADA and Shigeo KUBO

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co., LTD.*)

1. On the previous paper authors had reported the classification by antagonisms, on this paper, authors have reported the classification by serological properties.

2. Antigens of strains of *St. griseus* group and *St. olivaceus* were precipitated by own antiserum of each strains, and it was very interesting that the good precipitation was observed between *St. griseus* and *St. olivaceus* each other.

3. By way of exception, antigens of typical *St. griseus* eg. W#1, S-1 and other type cultures were not precipitated by antiserums of grisein-producing strains.

4. Antigens of *St. flavovirens*, *St. griseolus* and *St. scabis* which were lysogenized by actinophage of *St. griseus* were not precipitated by antiserums of *St. griseus* group.

5. From these results authors have guessed that *St. flavovirens*, *St. griseolus*, *St. scabis* and grisein-producing *St. griseus* may not identical with typical *St. griseus*, and *St. olivaceus* should be the same species as *St. griseus*.

6. According to authors studies on the fluctuation of serological properties in mutagenic processes, the serological properties were considered as a very stable key in classification.

Studies on the Classification of *Streptomyces griseus* Group. Part VII. Institution of Species Group and Some Criticism to Keys (p. *48～53*)

By Yujiro HARADA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co., LTD.*)

1. On this paper, author has tried to criticized to keys by a view point of stability in the variety of the source of isolation, many progeny and mutagenic processes.

2. From many experimental data of *St. griseus* group described on previous papers, it was reasonable to conclude that the morphological specification should be the 1st key, the serological properties the 2nd key, the carbon utilization spectrum the 3rd key, the actinophage sensitivity the 4th key, and the cultural properties eg. colors of aerial hyphae, soluble pigment, and some physiological properties, the antagonisms and the specification of spores should be written in wide scope description at the 1st key as a accessory.

3. According to author's arrangement of keys, author

has tried to institute a species group named as *St. griseus* group.

4. This species group was constituted by two species of *St. griseus* include Waksman's original line, S-1 line, several type cultures, KYOWA-Z-line, grisein-producing line, *rhodochrous* line and *St. olivaceus* include type culture, several vitamin B_{12}-producing strains.

5. The studies of genetic transformation between *St. griseus* and *St. olivaceus* will be reported later on.

Studies on Esterase from seeds. Part III. Estimation of Esterase-Activity (2). (p. *53～59*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It has been found in the previous experiment that the authors cannot use *p*-nitrophenyl acetic acid (*p*-NPA) as a substrate of embryo-esterase causing vividly spontaneous hydrolysis in the buffer solutions at pH values above 8.12.

Then, they have tried here to examine the salicylic acid (SA) method in order to be able to have experiments in various alkaline solutions excluding this inconvenience.

In this experiments, the stability of acetyl salicylic acid (ASA) in each buffer solution was estimated by the ultraviolet absorption spectra.

The experiments mentioned above resulted as follows:

(1) SA in each buffer solution was extremely stable.

(2) ASA in each buffer solution at pH values above 9.3 was extremely unstable. Consequently, they could not use ASA as a substrate under this condition. But ASA in the strong acid solution was very stable.

(3) They could use ASA as a substrate, in the case of enzyme reaction not lasting for long time in each solution at pH values near 7.0.

(4) Aqueous solution of ASA was not suitable as the stock solution, but methanol solution was very stable.

(5) In each alkaline solution the stability of ASA was higher than that of *p*-NPA.

Studies on the Activities of the Baker's Yeast. Part IV. Relationship between Maltose Fermenting Activity and Bread Making Activity. (1) The Accelerating Action for Maltose Fermentation of Wheat Flour. (p. *59-65*)

By Tomotaro SATO and Yasuo TANAKA

(*Food Research Institute, Ministry of Agriculture and Forestry*)

To find to relationship between the maltose fermenting activity and the bread making activity of baker's yeast, the accelerating influence on maltose fermentation of wheat flour was studied.

Japanese baker's compressed yeasts have only a negligible ability to ferment maltose, but they become to ferment it considerably in the medium containing wheat flour. Wheat flour contains accelerators on maltose fermentation and the amount varies with different flours and fraction of flours. Fractions which have much ash were found to be highly potent for the catalytic action, but the influence of bran was not so powerful as expected.

The accelerators on maltose fermentation of flour were found to be consisted of not only sugars, also nitrogenous matters, vitamins, and mineral matters. Among them nitrogenous matters have the most powerful influence.

Studies on the Activities of the Baker's Yeast. Part V. Relationship between Maltose Fermenting Activity and Bread Making Activity. (2) Maltose Fermentation and Dough Fermentation. (p. *65～68*)

By Tomotaro SATO and Yasuo TANAKA

(*Food Research Institute, Ministry of Agriculture and Forestry*)

The relationship between the maltose fermentation and dough fermentation was studied. The results of experiments are summarized as follows:

(1) The maltose fermenting activity of Japanese baker's yeasts varies considerably, but the variation does not effect on the dough fermenting activity.

(2) To have further information about the influence of maltose fermenting activity on dough fermentation, baker's yeast was inoculated in maltose-containing medium and was let ferment violently. The yeast become to have greater affinity to maltose but the same to glucose. The yeast effected favourably on dough fermentation, especially on the dough in which no sugar was added.

(3) Wheat flour contains so active accelerators on maltose fermentation that yeasts which are almost incapable of fermenting maltose become to ferment maltose considerably in dough. Nevertheless, it is found that the maltose fermenting activity is one of the very important characteristics of baker's yeast.

Studies on the Effect of Homogenizing Casein Solutions on the Properties of Casein. (p. *68～71*)

By Rinjirô SASAKI and Masahiro IWAIDA
(*Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo*)

The authors reported previously that when milk was processed under homogenization procedure the spherical form of casein was damaged⁽¹⁾. It is known that in milk itself casein exists in the form of micelles with calcium phosphate, the sizes of which ranging from 40 to 280 mμ⁽²⁾.

In these experiments many kinds of casein solutions were prepared, such as 3% Na-caseinate solution, 3% Ca-caseinate solution, 3% casein chloride solution and so on. Each solution was homogenized at 2,000 3,000 and 5,000 lb p.s.i. in a Gaulin homogenizer, and was subjected for the measurements of viscosity, tyrosine value, sulfhydryl group and peptic digestability, which were compared with the data previously obtained with calcium-phospho-caseinate⁽¹⁾.

From these results it became clear that effect of homogenization was largest when casein was homogenized in the shape of calcium-phospho-caseinate, secondly large with Ca- and Na-caseinate solutions and was smallest with casein chloride solution. No significant difference was observed between Ca- and Na-caseinate solutions.

(1) R. Sasaki and M. Iwaida: *J. Agr. Chem. Soc. Japan*, **32**, 752 (1958)
(2) H. Nitschmann: *Helv. Chim. Acta*, **32**, 1258 (1949)

Change in Sugar Contents during the Germination of Rice Seeds. Application of the Ion Exchange Resin Chromatography. (p. *72~78*)

By Toshio FUKUI and Ziro NIKUNI
(*Institute of Scientific and Industrial Research, Osaka University*)

Sugar contents of three organs (endosperm, sprout and root) of germinating rice seeds were estimated by means of ion exchange resin chromatography and paper partition chromatography.

1) Main sugar component of embryo and endosperm of non-germinating rice seeds was sucrose, and small amounts of raffinose, glucose and fructose were also found.

2) Raffinose disappeared completely in sprout and root of germinating seeds, but the amounts of sucrose, glucose and fructose increased. The ratio of these three sugars was maintained constant during the germination.

3) The amount of glucose of endosperm of germinating rice seeds increased markedly and reached maximum at 8tn day of germination (500 times of its original amount). Maltose and sucrose also increased and reached maximum at 8th day. Maltose was found only in the case of germinating endosperm. An unidentified oligosaccharide being composed of glucose and fructose was also found in later stage of germination.

4) Excised rice embryos were cultivated in darkness during 2 days on media containing one of the following 4 sugars: sucrose, glucose, fructose and maltose. The sprouting embryos contained sucrose, glucose and fructose in the same ratio as was found in the case of ordinary sprout and root, irrespective of their sugar sources.

Particle Weight of the Silk Fibroins by the Simple Osmometer. (p. *78~81*)

By Kei KOBAYASHI
(*Faculty of Agriculture, Kyushu University*)
and Miyo TANAKA
(*Faculty of Education, Saga University*)

A simple and inexpensive instrument for measuring the osmotic pressure reported by R.A. Mock et al. has been improved. It consists of two 1.2 mm I.D. capillary tubes sealed into a flanged glass sleeve, which is ground to fit snugly into a 20×60 mm round based cylindrical filter paper made by TŌYŌ ROSHI Co. designed No. 84. A generated cellulose membrance is cast over the cylindrical filter paper.

As the estimation for practical use of this apparatus, the molecular weight of horse hemoglobin has been determined and found to be 67,000—68,000, and it is entirely satisfactory for the osmotic determination of molecular weight.

And furthermore, for the applicational investigation of this apparatus, the particle weight of silk fibroin, which was prepared by Coleman and Howitt method using cupri ethylene diamine, has been determined. The authors found that the value of osmotic pressure was increased with treating hour of cupri ethylene diamine, therefore, particle weight was decreased. Although in this case extrapolating method was not proved for the best way, the ultimate value of particle weight at zero time of treating hour has been estimated, and found to be 74,000.

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Studies on the Requirements of Amino Acids and Vitamins. Part VII. Relation between Assimilable Nitrogen and Several Strains of Yeasts. (p. *83~86*)

By Masahiro TAKAHASHI and Ryuichiro TSUGAWA

(Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo)

The authors studied on the relationship between assimilability of nitrogen source and yeast strains.

The test organisms used were *Saccharomyces sake*, *Sacch. charente* Ungern, *Sacch. cerevisias*, *Sacch. acidificans*, *Sacch. rouxiim*, *Sacch. bisporus*, *Shizosaccharomyces pombe*, *Torula*, *Rodotorula*, *Candida utilis*, *Torulopsis*, *Hansenula anomala*, *Pseudosaccharomyces*, *Mycoderma*, *Pichia*, *Mycotorula*, and *Hansenula schaneggii*.

The source of nitrogen used in this experiment were NH_3 and casein hydrolyzate.

Some Assumption of Molecular Size of Degradation Products of Protein in Food Stuff with Ion Exchanger. Part I. Assumption of Colloidal State of "Miso". (p. *86~90*)

By Akira KAWAMURA, Kiso AKABANE and Fukue INAGAKI

(Department of Agricultural Chemistry, Tokyo Noko Daigaku, Fuchu, Tokyo, Japan)

For the purpose to study the degradation process of Soy bean protein in "Miso", the sieve analyses using Polystyrene Sulfonates were applied to the water soluble nitrogen in "Miso" which was different in ripeness.

At first, after NaCl in the samples being removed by electrodialysis with ion exchange membranes (Amberplex A-1, Amberplex C-1), the solutions were passed through Polystyrene Sulfonate columns having different crosslinking (DVB 1, 5, 10, 15%) then the total nitrogen remained in each effluent was determined.

Having been compared the data obtained from above experiments extending over 12 months with each other, we presume the degradation process of the Miso protein: the total water soluble nitrogen in "Miso" increases gradually within five months after mashing, then remains almost constant. And moreover the particle sizes of the decomposed products change themselves to the lower peptides and amino acids with aging especially beyond five months.

Next, in order to ascertain the about molecular sizes of the soluble part adsorped to each exchanger, Van Slyke's nitrogens in every fraction of the columns were also determined before and after acid hydrolysis.

Then we know that the water soluble part in "Miso" is consisted of very low peptides (Tri±or dipeptides) or amino acids and does not contain high peptides unexpectedly.

On the Mechanism of Urea-Utilization by Ruminants. Part IV. No Occurrence of Ammonium Carbamate during Urea Hydrolysis by Urease. (p. *91~96*)

By Masami SOEJIMA, Kiyoshi SUGAWARA, Eiichi KIKUCHI and Kensuke SHIMURA

(Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University)

A dilatometer of new type was devised to follow urease activity at an initial stage of hydrolysis of urea. When one mole of urea in phosphate buffer solution (pH 7.0) was hydrolyzed by urease, the volume contraction of the reaction system was found to amount to 21.0±1.5 ml at 35°C. On the other hand, the volume contractions produced by hydrolysis of urea to ammonium carbamate and ammonium carbonate were estimated to be 12.1 and 21.5 ml/mole, respectively, from the difference of apparent molar volume between amides and ammonium salts of monocarboxylic acid such as formic, acetic, propionic and carbonic acids.

These results seem to exclude a possibility of occurrence of ammonium carbamate as an intermediate of urease reaction. The stability of urea, carbamate and their related compounds were discussed from their infrared spectra and PK values.

On the Mechanism of Urea-Utilization by Ruminants. Part V. Decomposition and Formation Carbamyl Phosphate in Aqueous Solutions. (p. *97~100*)

By Masami SOEJIMA, Kiyoshi SUGAWARA and Kensuke SHIMURA

(Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University)

Recently Jones, Spector and Lipmann reported that carbamate formed during the decomposition of carbamylphosphate in Tris buffer at pH 7.0 was rather stable1). As this finding was contradictory to the authors' result of Part IV in the series of this study, a further investigation has been performed.

The apparent equilibrium constant of the addition reaction of potassium cyanate to primary potassium phosphate was found to be 1.38 ± 0.1, which give the following thermodinamic constants for the reaction: $\Delta F = -210$ cal/mole, $\Delta H = 5{,}000$ cal/mole and $\Delta S = 16$ cal/deg.

Carbamylphosphate decomposed spontaneously in an aqueous solution at pH 7.0 to phosphate and cyanate, not to phosphate and carbamate as Jones et al. reported. In the case of enzymic decomposition of carbamyl phosphate, ammonium, carbonium and phosphate ions were detected, but no carbamate.

1) M. E. Jones, L. Spector, and F. Lipmann; Proc. 3rd Intern. Congr. Biochem., Brussels. p. 278 (1955).

Chemical Studies on the Autolysis of Meats. Part X. On the Changes of Myoglobin and its Derivatives during the Ageing of Meats (1).

(p. *101~104*)

By Masao FUJIMAKI and Reiko OKURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo and Canners Association of Japan*)

Myoglobin from several kinds of meats was determined colorimetrically. Changes of myoglobin and its derivatives during the aging of meats were studied from the results of absorption curves in water extractive solution of aged meats.

Chemical Studies on the Autolysis of Meats. Part XI. On the Changes of Myoglobin and its Derivatives during the Ageing of Meats (2).

(p. *104~110*)

By Masao FUJIMAKI and Masako HASEGAWA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo and Faculty of Home Economics, Ochanomizu University*)

(1) Method of extraction of nitroso haem pigment from heated cured meats was studied.

(2) The content of nitroso haem pigment extracted from heated cured meats changed from the period of curing, and cured meats showed the maximum value at two to four days after curing.

(3) Effects of curing materials upon the fixation of meat color in cured meats and its mechanism were studied.

(4) Optimum condition for the fixation of meat color in hot curing, as used in the curing of canned corned beef was studied. Fixed meat color in hot curing seemed to be slightly inferior to the one in normal (cold) curing from the determination of nitroso haem pigment.

On the Rate of Mutarotation of Lactose and β-Galactosidase Action on Lactose Isomers.

(p. *111~116*)

By Rinjiro SASAKI and Kôkichi TANIGUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

It is well known that α- and β-lactose differ in some physical and chemical properties. Accordingly, the authors wanted to analyze their enzymological properties. Intestinal mucosa and yeast β-galactosidase were used in this experiment.

It is unavoidable that these two lactose isomers interconvert, each other in solution, finally forming equilibrium state. Firstly, the specific rotative powers of α-, β- lactose and lactose at equilibrium state were determined at different temperature, secondly, the rate of interconversion between two isomers was erucidates at different pH and temperature. These values are summarized in tables. From the above results, it became clear that the ratio of α- and β-lactose at equilibrium state tends to decrease with increasing temperature and the rate of interconversion between the isomers shows a minimum in the range of 3.0–5.0.

The differences of β-galactosidase action on two isomers of lactose was studied at 25°C, pH 5.0 for 20 minutes when the rate of interconversions was rather small and had not to be taken into account. β-Galactosidase has greater affinity to α-lactose than to β-lactose.

Studies on the Proteolytic Enzymes of Molds. Part XV. Analysis of Fungal Protease Systems by means of their pH-Activity Curves and of Inhibitors.

(p. *116~120*)

By Kin'ichi MATSUSHIMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Mie University*)

The protease systems of different molds were analyzed by means of the character of their pH-activity curves and by means of inhibitors. Three kinds of inhibitors, i.e. Na-laurylsulfonate, EDTA, and potato-inhibitor, were used in this experiment, and the extent of proteolysis and its inhibition at various pHs were determined by the method of Anson: milk casein was used as substrate. As the result, the following proteases were identified as the constituent of the protease system of each mold; acid-, semi-acid-, and alkaline protease for *Penicillium chrysogenum*, acid-, and semi-acid protease for *Rhizopus javanicus* as well as for *Penicillium luteum*, and singly neutral protease for *Hormodendrum* sp.. Of the proteases, above described, semi-acid protease, which has optimal pH at 7 and is inhibited neither of the inhibitors, was the one which is discovered for the first time as the result of this experiment.

Studies on the Proteolytic Enzymes of Molds. Part XVI. Protease System of Taka-diastase. (p. *120∼125*)

By Kin'ichi MATSUSHIMA

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The proteases in Taka-diastase were devided into two fractions by rivanol, and the protease constituent of each fraction was analyzed in a similar manner as in the experiment of the previous report. Acid-, semi-acid-, and alkaline protease were detected in the fraction not precipitated with rivanol. The fraction precipitated with rivanol was found to be composed singly of neutral protease which lost its activity by metal chelating reagent, EDTA.

Environmental Variation of the Chemical Constituents of the Tea Leaf. Part IV. Distribution of Nitrogenous Fractions in the Tea Seedling Grown in Dark. (p. *125∼128*)

By Hideichi TORII and Isao ÔTA

(*Tokai-Kinki Agricultural Experimental Station, Tea Division*)

The nitrogenous fractions of etiolated young tea seedling grown in a dark room were compared with those of normal seedling. Caffeine was not found in the seed and subterranean parts of young seedling, but found in overground parts grown both in dark and light. Its content was several folds higher in the etiolated seedling than in the normal one. The amounts of soluble and insoluble nitrogen in the etiolated seedling were the same as in the seed, but in the normal seedling about one-third of the soluble nitrogen in the seed turned to insoluble form and one-tenth to caffeine, the larger parts of which were accumulated in the leaf. Theanine, the specific amide in tea, was the most abundant in the root, and glutamine was detected in the seedling grown both in dark and light, while asparagine only in dark.

Studies on Egg Yolk Proteins. Part I. Ultracentrifugal Behaviours of Lipoproteins from Hen's Egg Yolk. (p. *128∼133*)

By Kiyozo HASEGAWA and Hisateru MITSUDA

(*Laboratory of Nutritional Chemistry, Faculty of Agriculture, Kyoto University*)

Ultracentrifugal examinations have been carried out on the lipovitellin and the lipovitellenin fractions prepared from the hen's egg yolk according to the method of Fevold et al.. It has been indicated in the 10 per cent sodium chloride solutions that the lipovitellin fraction consists of three sedimenting components, and that the lipovitellenin fraction consists of one floating component. The sedimenting components of the lipovitellin fraction were numbered I, II, and III in order beginning with the most rapid. The appearent relative protein concentrations of the component I, II, and III were about 10, 55, and 35 per cent, respectively. The values of the sedimentation constants extrapolated to zero concentration, $S_{20,w}{}^{0}$, were 11.1 S for the component II and 5.18 S for the component III, respectively.

Studies on the Auto-toxication of Bacterial Cultures. (p. *133∼137*)

By Ooki NAKAYAMA and Seiji TADA

(*Agricultural Department of Tamagawa University, Tokyo*)

By means of B. coagulans, *E. coli* and *L. casei*, the present authors showed the following phenomena:—

1. In a definite species and cultural condition, sizes of bacterial colonies on a solid media are roughly in reverse proportion to the colony number per a plate.
2. Bacterial cell number per unit quantity of broth media increases by dipping an agar block into the broth. The extreme case is the agar culture; then, bacterial cells are packed compactly.
3. "Two agar layers cross streak method" gave the results that neighbourhoods of a bacterial colonies are especially unfavourable to the growth of the same species of bacteria.

From above mentioned phenomena, the present authors infered that bacteria excrete certain auto-toxicating substances, tried to extract the substances. They discussed the autotoxicating phenomena relating with the "Protomorphogen" hypothesis.

Determination of Chromic Oxide and Barium Sulfate used as a Trace Material for the Movement of Intestinal Contents.

(On the Methods for the Study of Digestion and Absorption. Part II) (p. *138~141*)

By Ichiro YOSHIHARA

(*Faculty of Agriculture, University of Niigata*)

For the study of the movement of gastrointestinal contents, chromic oxide and barium sulfate have been used. Injections of chromic oxide and barium sulfate into different parts of digestive tract will offer more useful data.

The method for determination of chromic oxide and barium sulfate in feces by the use of a single fused mass was studied.

Chromic oxide, barium sulfate and ash of feces of a rabbit were placed in Ni-crucible. After additions of 5 g. of anhydrous sodium carbonate and 5 g. of sodium peroxide, fusion was continued for 20 minutes with nearly full flame. The contents were washed out with water and filtered. Chromate in the filtrate was assayed titrimetrically and barium carbonate in the precipitate on the filter paper was dissolved with dilute hydrochloric acid and determined by means of a direct isotope dilution method used ^{140}Ba.

Recoveries of chromic oxide and barium sulfate were satisfactory.

The Effect of Rennet on Lactic Acid Bacteria in Cheese Ripening.

(p. *142~147*)

By Umeo YOSHINO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The effects of rennet on the acid production and proteolysis of milk protein by lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Streptococcus cremoris*, *Streptococcus lactis* and *Streptococcus thermophilus*) inoculated in milk were investigated.

The results indicate that the acid production by most of the lactic acid bacteria examined was depressed by the addition of rennet to milk, and none of the test organisms was stimulated to produce acid by rennet. The apparent inhibitory effect of rennet is due to the fact that in spite of the stimulatory ability of the hydrolyzed products of casein by rennet, casein is changed to paracasein which is found to be less available to lactic acid bacteria.

The opalescent material(1) produced in sodium caseinate solution by rennet has a stimulatory effect on the acid production by lactic acid bacteria.

The increased proteolysis by lactic acid bacteria was observed in milk subjected to rennet action.

The experiment on the effect of pepsin on lactic acid bacteria gave the similar results as in the case of rennet.

(1) U. Yoshino, *Bull. Agr. Chem. Soc. Japan*, **22**, 242 (1958).

Studies on the Nutrients to be regarded when the Rat Content is increased in the Diet. Part III.

Change of Requirement of Tocopherol and Methionine as influenced by the Increase of Fat in the Diet. (p. *147~153*)

By Tatsuo KOYANAGI, Harunobu NORO, Kazue KUNIMOTO and Kazuko KAMADA

(*Faculty of Agricultural Chemistry, Iwate University*)

The addition of linoleic acid methylester to the rice diet which contained 5% casein resulted the liver necrocis and hypertrophy of adrenal and spleen of rats. The liver damage and hypertrophy of the organs was prevented by dl methionine uncompletely and by a tocopherol completely.

(*continued from front cover*)

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